Analysis of Flavan-3-ols by Capillary Electrophoresis

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Abstract

People in France have a lower mortality rate from coronary heart disease when compared with other western countries. Some scientists have proposed the intake of red wine as explanation for this finding. Red wine contains flavan-3-ols, which can produce antioxidant action in preventing coronary heart disease. The flavan-3-ols in red wine come from the grape seed. As such, grape seed extract has been gaining its importance over the decade as a health supplement.

In the literature, the separation and detection of flavan-3-ols using HPLC and some colorimetric methods have been reported. But these methods have disadvantages such as low resolution and poor reproducibility.

In the present study, an analytical method using capillary electrophoresis has been developed to analyze flavan-3-ols derivatives, and it was subsequently applied to study the quality of grape seed extracts obtained from commercial sources. Analytical conditions such as buffer concentration, surfactant concentration, cyclodextrin concentration and the presence or absence of organic modifier were optimized. Eight reference compounds, which could be successfully resolved, were

adopted.

The method developed for the analysis of grape seed extract is simple. The time for analysis is short and only a little of amount of sample is needed. It also has good reproducibility. This method is considered to be a suitable method for qualitative and quantitative analysis of grape see extract.

摘要

統計發現法國人在冠心病的死亡率比其他西方國家爲低。有些科學家認爲出現這現象的原因是法國人每天都飲用紅酒。紅酒裏含有 3-黃烷醇，它是一個抗氧化劑。紅酒裏的 3-黃烷醇來自葡萄。所以，葡萄籽提取物在近十年的健康食品市場中佔有重要的位置。

在文獻紀錄中，3-黃烷醇的分離多採用高效液相色譜法和比色法。但這些方法有一定的缺點，如解像度低和重現性低。

在此項研究中，利用毛細管電泳檢測 3-黃烷醇成份，並用以不同來源的葡萄籽提取物。緩沖液的組成和濃度，表面活性劑濃度，環糊精濃度和有機改性劑加入量都做到了優化。在最後的優化條件下，成功分離了八個標準品。此方法並應用於不同來源的葡萄籽提取物。

利用本方法來測定葡萄籽提取物的好處是簡單、省時、需要的樣品量少和具有良好的重現性。所以，這方法可用來作爲葡萄籽提取物的定性和定量分析。

Table of Contents

Abbreviations

C	(+)-catechin
CD	cyclodextrin
CE	capillary electrophoresis
CG	(-)-catechin gallate
EC	(-)-epicatechin
ECG	(-)-epicatechin gallate
EGC	(-)-epigallocatechin
EGCG	(-)-epigallocatechin gallate
EOF	electroosmotic flow
GC	(-)-gallocatechin
GCG	(-)-gallocatechin gallate
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
LDL	low density lipoprotein
MEKC	micellar electrokinetic chromatography
MS	mass spectrometry
SDS	sodium dodecyl sulfate

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Chapter 1

Introduction

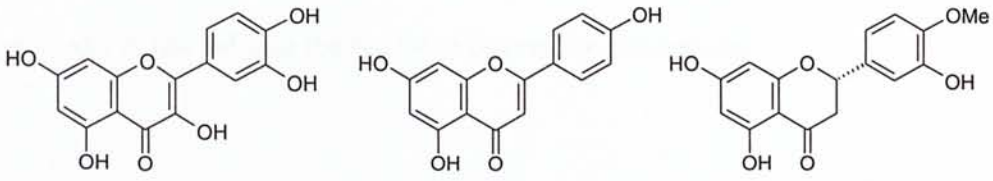
1.1 The French Paradox

In most developed countries, there is a high dietary intake of saturated fat and cholesterol because of large consumption of butter, cheese, eggs, etc. Epidemiological studies have shown that these kinds of diet positively correlate with mortality from heart disease [1]. In France, 15% of the daily calories were from saturated fats. However, the mortality rate from coronary heart disease in France is only about 40% of other developed countries. This paradoxical finding is known as the French Paradox [2-4].

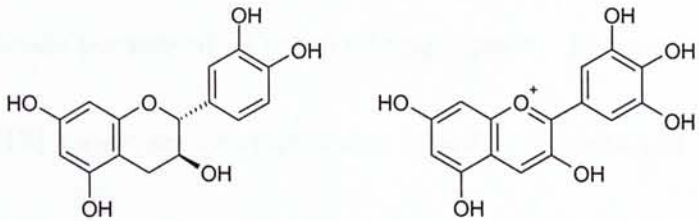
Scientists have been trying to find the reason to explain why the French have a lower incidence of coronary heart disease than expected. One distinctive feature of the French diet is their regular consumption of red wine with meals. Researches have shown that people who had consumed three to five glasses of wine per day for 12 years had only half the mortality risk than those who did not consume wine [5].

1.2 Flavonoids

Red wine contains water, alcohol, ascorbic acid, sulfur dioxide, carbon dioxide, tartaric acid and flavonoids etc. Flavonoids are a group of polyphenolic compounds found in plants. More than 4000 chemically unique flavonoids have been identified in plant source and in beverages [6]. Five basic subclasses of flavonoids have been recognized: flavones, flavan-3-ols, flavanones, flavonols and anthocyanidins [7]. A flavonoid molecule consists of two benzene rings connected by a three-carbon chain. In most of them, the chain is closed by an oxygen, forming a third ring [6]. Examples of the structures of different subclasses are shown in Figure 1.1:



Flavonol-Quercetin Flavone-Apigenin Flavanone-Hespercetin



Flavan-3-ol-catechin Anthocyanidin-Delphinidin

Figure 1.1 Structures of selected examples of the five subclasses of flavonoids

Selected flavonoids have been shown in numerous in vitro and in vivo experiments to have antiallergic, [8] anti-inflammatory, [8] antiviral, [8] anticancer [8] and antioxidant activities [9-13]. It also inhibits the copper-catalyzed oxidation of low-density lipoprotein (LDL) [14-16]. Antioxidant capacity of human blood plasma increased after the oral intake of polyphenols and a decreased level of LDL cholesterol oxidation has been observed [14-15]. One of the major kinds of flavonvoids present in red wine is flavan-3-ols [1]. They are originated from the

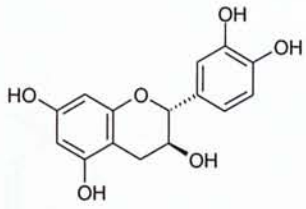
grape seeds, extracted into the red wine during the production.

1.3 Grape seed extract

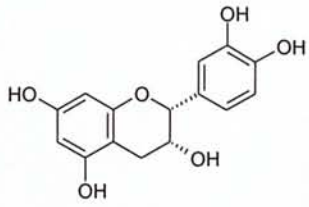
Grape seed extract has been gaining its importance as a health food supplement over the last decade because of its antioxidizing capacity. In Japan, it is an approved food additive [17]. Grape seed extract is also added in cosmetics as an antioxidant to delay the aging process. Grape seed extract has been shown to be non-toxic in acute oral toxicity study, acute dermal toxicity study, primary dermal irritation study, as well as primary eye irritation study in albino rabbits [18]. It was also shown to have greater antioxidative effect than vitamin E, C, β-carotene, and a combination of vitamin C plus E [18].

The dried grape seed extract is a reddish brown powder. It is soluble in polar solvents such as water, methanol, ethanol, acetone, but not soluble in non-polar solvents such as petroleum ether and benzene [19].

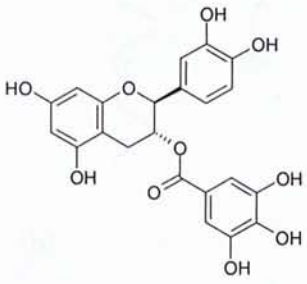
Flavan-3-ols is a kind of antioxidant commonly found in grape seed extracts. Several naturally occurring flavan-3-ol structures are shown in Figure 1.2:



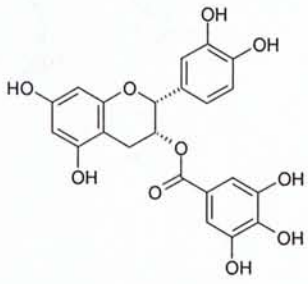
(+)-catechin



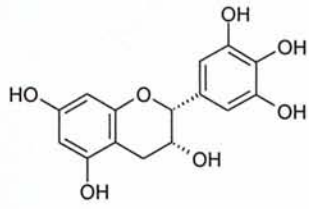
(-)-epicatechin



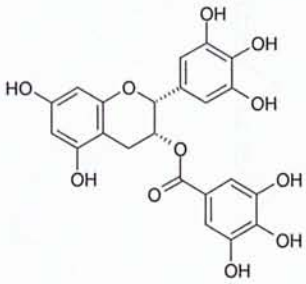
(-)-catechin gallate



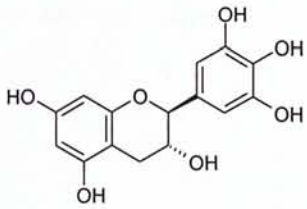
(-)-epicatechin gallate



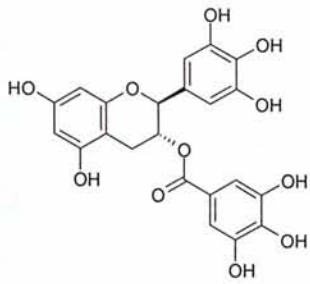
(-)-epigallocatechin



(-)-epigallocatechin gallate



(-)-gallocatechin

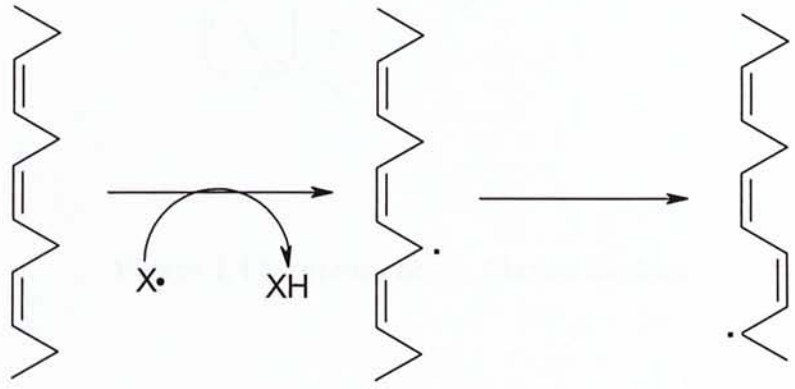


(-)-gallocatechin gallate

Figure 1.2 Structures of common flavan-3-ols

Flavan-3-ols act as an antioxidant by donating electrons from the phenyl group to the free radicals and breaking the radical chains. They localize the resulting radical within the structure to form the less reactive flavonoid phenoxyl radical [Figure 1.3]. Flavan-3-ols also act as an antioxidant by metal ion chelation [20-21].

(a)



(b)

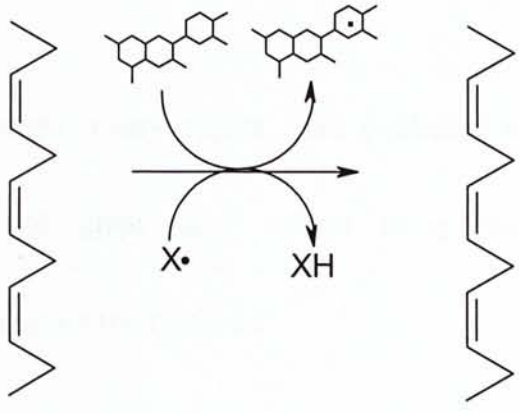


Figure 1.3 (a) Free radical attack of polyunsaturated fatty acid group present in LDL. (b) catechin stabilization of the free radical protecting the polyunsaturated fatty acid

The degree of hydroxylation of the B ring shown in Figure 1.4 is one of the factors that determine the antioxidizing capacity of the flavan-3-ols. The higher the degree of hydroxylations, the higher the antioxidizing ability.

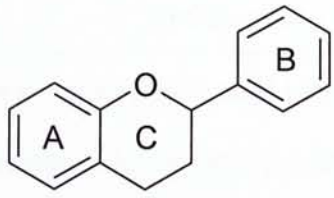


Figure 1.4 Structure of the Flavan nucleus

1.4 Instrumental analysis

In the past 20 years, many papers were published on the qualitative and quantitative analysis of grape seed extract using high-performance liquid chromatography and colorimetric methods.

1.4.1 High-Performance Liquid Chromatography

A number of studies have been published on the characterization of grape seed extract using different stationary and mobile phases to separate flavan-3-ols and proanthocyanidins. Most of these studies employed reverse phase HPLC [17, 22-25]. Both UV [25] and MS [22-24] have been used as the detection mode. Some separation conditions employing HPLC were shown in Table 1.1.

Stationary phase	Mobile phase	Detection
Develosil 300 ODS-HG-5	(A) water/phosphoric acid 1000:1 (v/v) (B) acetonitrile/phosphoric acid 1000:1 (v/v) 0-20min, 100-85% A, 0-15% B; 20-25min, 85-70% A, 15-30% B; 25-45min, 70-0% A, 30-100% B; 45-61min 0% A, 100% B	Fluorescence [17]
Phenomenex Luna 5 μm Silica column (250 X 4.6 mn)	(A) dichloromethane (B) methanol (C) acetic acid/water 1:1 (v/v) 1-30 min, 82-68% A, 14-28% B; 30-60 min, 68-48% A, 28-50% B; 60-65min, 46-10% A, 50-86% B; 65-70min, isocratic. C keep constant at 4%	UV and MS (negative APCI mode) [22]
Zorbax XDB (5μm, 4.6mm X 250mm)	(A) 1% formic acid (B) 80% methanol 1% formic acid 0-5min, 100% A, 0% B; 5-50min, 100-20% A, 0-80% B,	ESI-MS [23]
Zorbax SC-C_{18} (250 X 4.6mm)	(A) 1% acetic acid (B) 99% actonitrile 1% acetic acid 0-20min, 95-87% A, 5-13% B; 20-30min, 87% A, 13% B; 30-46min, 87-78% A, 13-22% B; 46-55min, 78-10% A, 22-90% B; 55-65min, 10% A, 90% B	UV and MS (negative APCI mode) [24]

Exsil 100 5μ ODS (4.6 X 250mm)	(A) 0.2% phosphoric acid (B) 82% acteonitrile, 0.04% phosphoric acid 0-15min, 100-85% A, 0-15% B; 15-40min 85-84% A, 15-16% B; 40-45min 84-83% A, 16-17% B; 45-48min 83-57% A, 17-43% B; 48-49min 57-48% A, 43-52% B; 49-56min 48% A, 52% B; 56-57min 48-57% A, 52-43% B; 57-58 min 57-83% A, 43-17% B; 58-60 min 83-100% A, 17-0% B	UV [25]

Table 1.1 Separation conditions of HPLC analysis

1.4.2 Colorimetry

The flavan-3-ols content in the grape seed extract can be determined by Vanillin-HCl assay [26-27]. When the grape seed extract solution is added to a mixture of 1% vanillin and HCl in methanol, incubated for 20 minutes at 30 ℃, the absorbance of the resulting mixture was then measured [17]. The reaction is based on the condensation of the phenolic aldehyde (vanillin) with the phloroglucinol structure of flavan-3-ols under acidic condition in an organic solvent. The resulting compound is red in colour [28]. However, this method lacks reproducibility between samples, days and laboratories [29].

1.5 Capillary electrophoresis

1.5.1 Instrumentation

The instrumentation required for capillary electrophoresis is simple [Figure1.5]. The ends of a capillary are placed in two buffer reservoirs, each containing an electrode connected to a high-voltage power supply. During sample loading, one of the buffer reservoirs is temporarily replaced by the sample vial. An electric potential or an external pressure is then applied for a few seconds. After switching back to the buffer reservoir, an electric potential is applied across the capillary and the separation is performed. Optical detection is performed directly through the capillary wall near the opposite end. The data are then expressed as an electropherogram with detector response versus migration time.

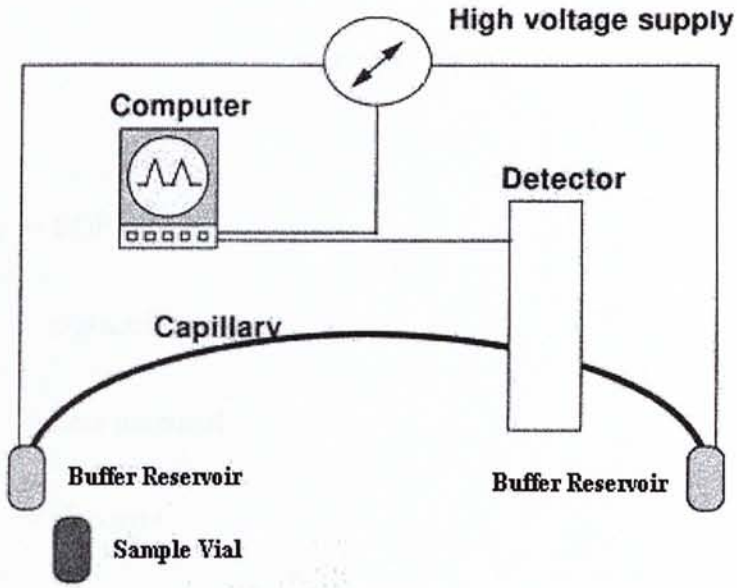


Figure 1.5 Typical instrumental setup for capillary electrophoresis

1.5.2 Electroosmotic flow

On the surface of the capillary, there are many ionizable silanol groups. When the silanol group is in contact with the buffer solution, the silanol group readily dissociated. The capillary surface thus became negatively charged. Negative charges on the capillary wall cause the building up of cations near the surface. As a result, an electric double layer is formed [Figure 1.6]. Upon application of electric field, the positive ions move towards the cathode, bringing the whole bulk of solution towards the detector. This is known as the electroosmotic flow (EOF) [30].

$$\mu_{eof} = (\epsilon\zeta)/\eta$$

where μ_{eof} = EOF mobility

ϵ = dielectric constant,

ζ = Zeta potential

η = viscosity

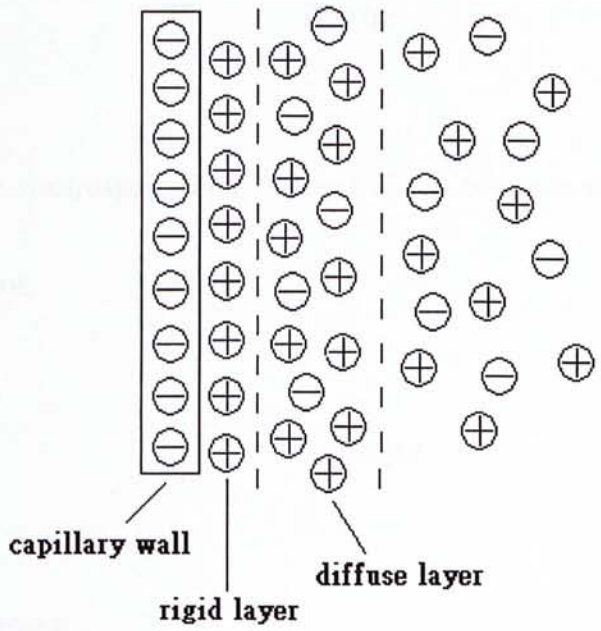


Figure 1.6 Representation of the electric double layer

1.5.3 Electrophoretic mobility

During the analysis, the capillary is filled with buffer solution. When a constant electric field (E) is applied across the capillary, the ionic species inside the capillary experience an electrostatic force (F_e), which is proportional to the electric field strength and the charge (q) of the particular ion. The electrostatic force cause the ion to move towards the oppositely charged electrode [31].

$$F_e = qE$$

Beside the electrostatic force, there is also a counteracting frictional force (F_f) acting on the ions.

$$F_f = -6\pi\eta rv$$

Where η = viscosity

r = radius of ion

v = velocity

After a steady stage is reached, the ions move with constant velocity

$$v = \mu_e E$$

where v = velocity

μ_e = electrophoretic mobility

E = the applied electric field

By solving the equations, electrophoretic mobility can be expressed as

$$\mu_e = q/6\pi\eta r$$

In overall term, the electroosmotic flow is uaually greater then the electrophoretic mobility. So cations, anions and neutral species will all reach the detector with the order of cations followed by neutral species and finally anions [Figure 1.7 and 1.8].

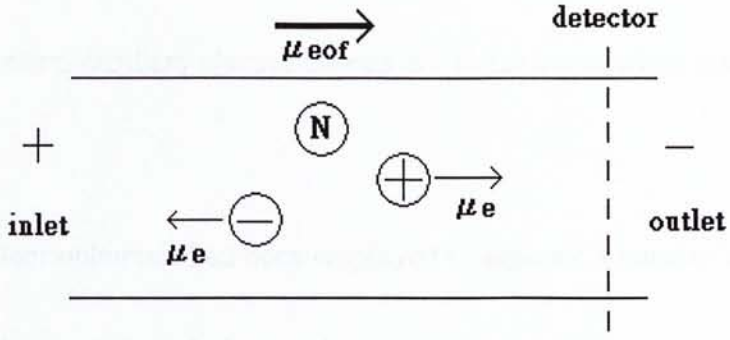


Figure 1.7 Overall view of movement of ions in capillary

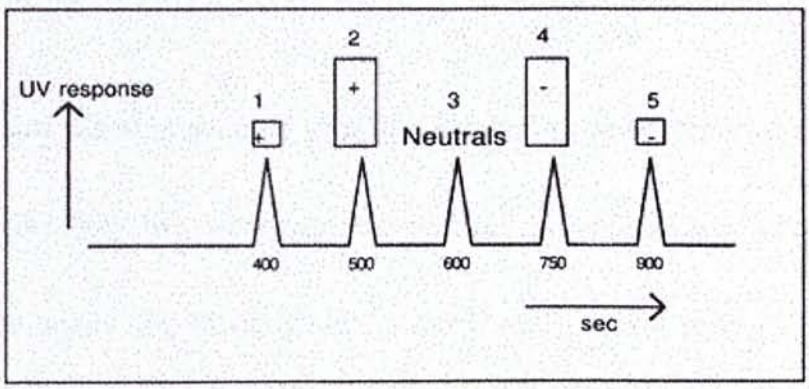


Figure 1.8 Representation of the order of elution in electrophoregram

1.6 Objective of the study

Capillary electrophoresis was chosen as the analytical method because of its high speed, high separation efficiency, high resolution, low risk of zone broadening, small sample volume, small volume of reagents and good reproducibility [32]. Since the

polyphenolic compounds in the grape seed extract have similar structures, some of them being epimers, capillary electrophoresis is a suitable analytical method.

Capillary electrophoresis had been employed to separate a number of compounds from plants. This includes alkaloids, glucosinolates, capsaicinoids, polyamines and terpenes. Among phenolic compounds, capillary electrophoresis had been used to separate coumarins, phenolic acids and quinines [33].

For the analysis of grape seed extract, a number of papers reported the analysis with HPLC but only two were reported with the use of capillary electrophoresis [34-35]. The major objective of the present study is to develop a capillary electrophoresis method for the analysis of grape seed extracts.

A number of samples of grape seed extracts were collected from commercial sources originated from different countries. The first part of the research project is to develop an analytical procedure with good repeatability and resolution by capillary electrophoresis. This is achieved by determining a method with optimized buffer concentration, pH, temperature and other operating conditions. The second part of the research project deals with the qualitative and quantitative determination of

flavan-3-ols in different grape seed samples. The method developed in the present study can be applied to the quality assurance and quality control of grape seed products.

Chapter 2

Experimental

2.1 Reagents and materials

2.1.1 Reagents

Sodium tetraborate-10-hydrate was obtained from Fluka-Garantie, sodium dodecyl sulfate from Riedel-de Maen, α-cyclodextrin, β-cyclodextrin (minimum 98%) and urea were from Sigma. γ-cyclodextrin was purchased from Wacker. Sodium hydroxide pelletes was obtained from Merck, whereas hydrochloric acid was from Mallinckrodt. All solvents used were of HPLC grade. Methanol, ethyl acetate and acetonitrile were purchased from Merck.

2.1.2 Instrumentation

All analyses were carried out on a HP ^{3D} capillary electrophoresis system. It consists of a variable voltage (0 to 30 kV) power supply, an on-capillary photodiode array (190 to 600 nm) detector, a capillary cassette and an air thermostatting system.

The polarity of the instrument was set to be positive. The detection was made at the cathode end. The capillary used was an uncoated fused silica capillary (Agilent). The total length of the capillary was 90 cm, with an effective length of 81.5 cm. The detection window was generated by fusing the polyimide coating of the capillary 8.5 cm from the outlet end. The residue was then removed with methanol and distilled water. Electrokinetic injection was used in all experiments. Data were collected and processed by the HP ^{3D} CE Chemstation software.

2.1.3 References compounds

(+)-Catechin and (-)-epicatechin were purchased from Aldrich Chem. Co. (-)-Epigallocatechin, (-)-catechin gallate, (-)-gallocatechin, (-)-epicatechin gallate, (-)-gallocatechin gallate were obtained from Wako, while (-)-epigallocatechin gallate was obtained from Sigma.

2.1.4 Samples

Seven samples were collected from different commercial sources, including Polyphenolics (product originated in USA), Trusperity USA inc. (product originated

in China), Scandinavian Formulas Chemical and Ingredients Division (product originated in Europe), DNP International Co. Inc (product originated in China), Future Ceuticals (product originated in USA), AMAX Nutritional Inc (product originated in China) and Nutratech (product originated in China).

2.2 Selection of solvent for sample preapartion

Grape seed extract is soluble in polar solvent. Although water, ethanol and acetone could extract flavan-3-ols from grape seed extract, many impurities are also extracted. Ethyl acetate was chosen as the solvent to extract flavan-3-ols from grape seed extract as it was a more selective solvent that could lower the amount of impurities [21, 27].

2.3 Procedures

2.3.1 Preparation of running buffer solution

Borate buffer was prepared by dissolving appropriate amount of sodium tetraborate-10-hydrate in deionized water. Accurate amount of sodium dodecyl sulfate, β-cyclodextrin, γ-cyclodextrin and urea were then added and dissolved in the buffer solution. The buffer solution was then sonicated for 30 minutes to assist dissolution and to remove air bubble. The pH of the buffer was then altered to the desired pH with 0.1 M sodium hydroxide and 0.2 M hydrochloric acid. pH meter (SCHOTT) was used for pH measurement. It was calibrated with two-point calibration, using standard pH 4.00 and pH 7.00 buffer solutions. The buffer solutions were filtered through 0.45 μm syringe filter before use.

2.3.2 Preparation of standard solution

Reference compound (0.0020g) was dissolved in 70% methanol solution in a 1-ml volumetric flask and stored under refrigeration (-20℃) to avoid decomposition. Standard solutions were filtered through a 0.45 μm syringe filter before injection into

the capillary.

2.3.3 Preparation of sample solution

Sample (1g) was weighed in a conical flask and extracted with ethyl acetate (15mL) [20]. The total weight was recorded. The sample was sonicated for 15 minutes and the total weight was then measured again. Ethyl acetate was added to recover the loss during extraction. The sample solution was then filtered. The filtrate (10mL) was collected and dried, redissolved in 70% methanol (2mL), and filtered through a 0.45 μm syringe filter before injection.

2.3.4 Flushing procedures

On the first use of the capillary, it was flushed with 0.1 M sodium hydroxide solution for 20 minutes, followed by deionized water for 30 minutes, and finally with the running buffer for 30 minutes. A blank run for 30 minutes was carried out before the first injection. Before the start of experiment each day, the capillary was flushed with 0.1 M sodium hydroxide solution for five minutes, followed by deionized water and the running buffer for 10 minutes each. At the end of experiment each day, the

capillary was flushed with 0.1 M sodium hydroxide solution for five minutes, followed by deionized water for 10 minutes, and finally with air for three minutes. In between runs, the capillary was flushed with 0.1 M sodium hydroxide solution for two minutes, followed by deionized water for three minutes, and finally with the running buffer for 10 minutes to ensure a stable condition is generated in the capillary before each run.

Chapter 3

Results and Discussion

3.1 Preliminary experiments

From literature findings [32], the separation of (+)-catechin and (-)-epicatechin was performed by using 0.1M borate at pH 9.2 as the running buffer. Borate was reported to be able to form complexes with flavonoid and negatively charged complexes were formed, thus the separation could be more effective based on charge-to-mass ratios of the deprotonated polyphenols or through borate-phenol association [33]. This buffer system was considered in our first trial experiment. The experimental conditions are shown below:

Buffer : 0.1M sodium tetraborate at pH 9.2

Capillary : 90cm X 50 μm i.d. uncoated fused silica capillary, with effective length of 81.5cm

Applied voltage : 25kV

Temperature : 20℃

Injection : electrokinetic injection 5kV for 3 seconds

Detection wavelength : UV detection at 220 nm

Under the above condition, (-)-catechin gallate and (-)-epicatechin gallate could not be separated and the resolution for the separation was poor [Table 3.1]. The migration time needed to complete the whole separation was more than 60 minutes. This condition was therefore considered not suitable for further experiments.

Reference compounds	Migration time (mins)
(-)-Epigallocatechin (EGC)	41.265
(-)-Epicatechin (EC)	42.248
(-)-Gallocatechin (GC)	43.434
(+)-Catechin (C)	44.426
(-)-Epigallocatechin gallate (EGCG)	58.15
(-)-Catechin gallate (CG)	60.075
(-)-Epicatechin gallate (ECG)	60.075
(-)-Gallocatechin gallate (GCG)	62.068

Table 3.1 Migration time of the eight reference compounds .

Buffer : 100 mM borate solution (pH 9.2)

The concentration of borate buffer (pH 9.2) was then changed to 50mM under the same conditions as in the previous experiment. The results are shown in the Table 3.2. The migration time was reduced significantly. When the ionic strength of the separation buffer decreases, the EOF increases. Thus, the separation time is shortened.

Reference compounds	Migration time (mins)
(-)-Epigallocatechin	25.246
(-)-Epicatechin	25.246
(-)-Gallocatechin	25.631
(+)-Catechin	26.084
(-)-Epigallocatechin gallate	31.274
(-)-Catechin gallate	31.274
(-)-Epicatechin gallate	31.836
(-)-Gallocatechin gallate	31.836

Table 3.2 Migration time of the eight reference compounds .

Buffer : 50 mM borate solution (pH 9.2)

3.2 Effect of pH

The pH is one of the most important factors in CE analysis. Increasing pH not only causes the electroosmotic flow to increase, but also increases the ionization of acids and reduces the ionization of bases. These changes are crucial factors in the determination of migration time.

The pH range from 7.0 to 9.2 was investigated [Figure 3.1]. The results showed that when pH of the buffer was decreased from pH 9.2 to pH 7, both peak shape and resolution were improved [Figure 3.2]. The migration time was significantly shortened as the pH value decreased. Being acidic in nature, flavan-3-ols are ionized under alkaline condition to give negative charges. The negative charges caused the analyte to move towards the anode. Hence, the time needed for the analyte to reach the detector (cathode) is increased at alkaine pH. It was also observed that the four reference compounds without gallate group migrated faster than the other four reference compounds containing gallate group. This is because the reference compounds with gallate group bear higher negative charges, thus retaining for a longer time in the capillary.

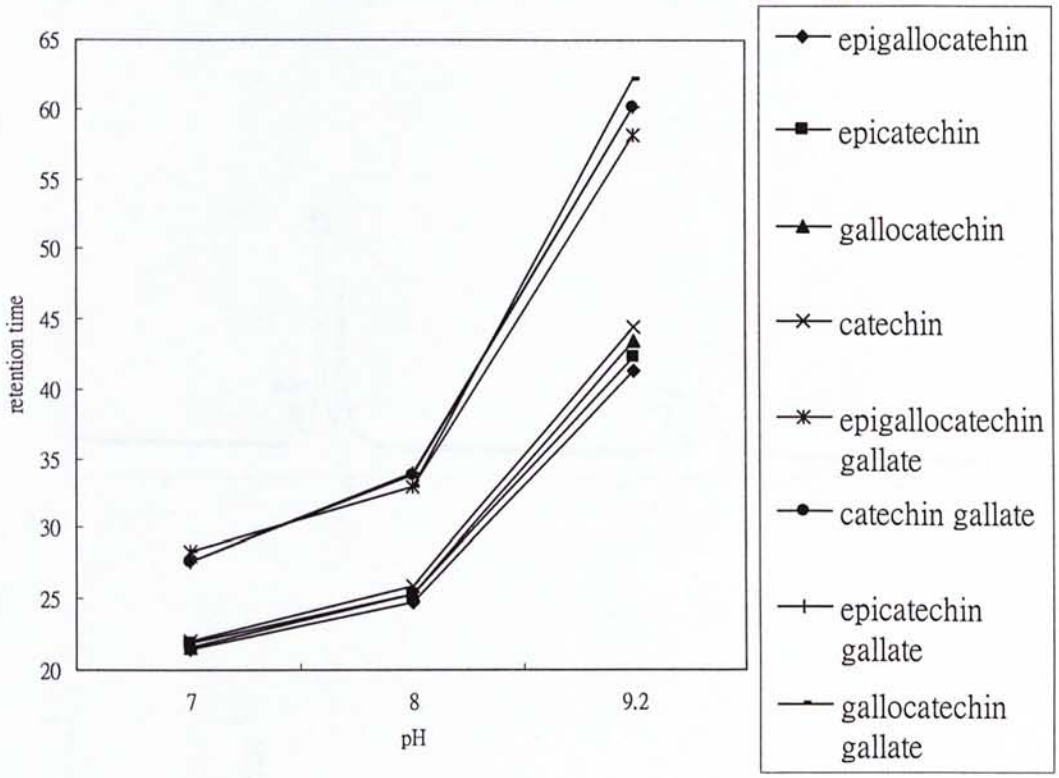
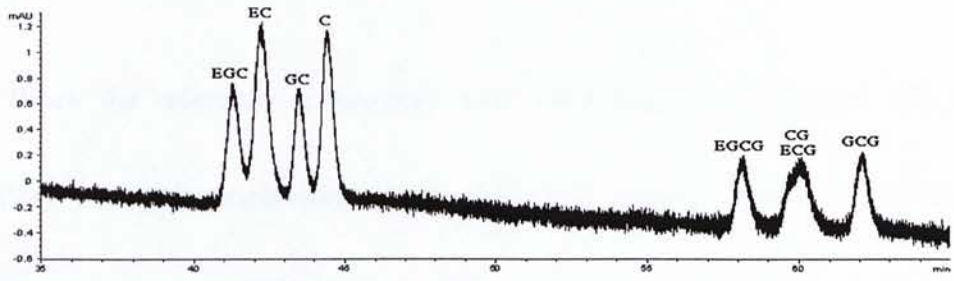


Figure 3.1 Effect of pH on migration time

Buffer : 100 mM borate solution (pH 9.2)

(a)



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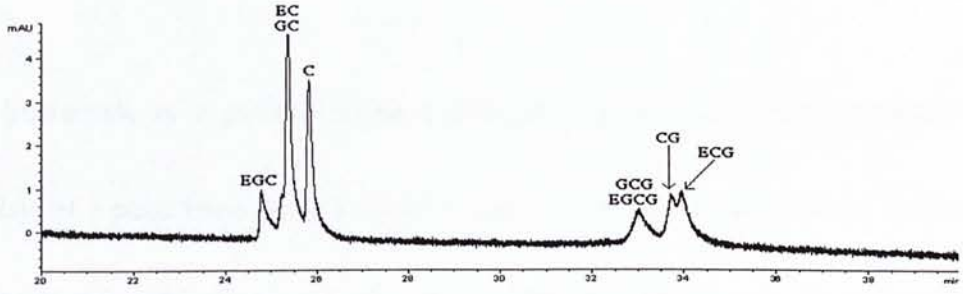

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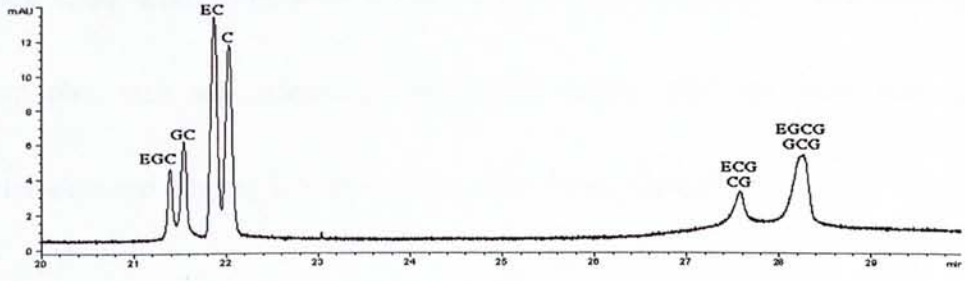


Figure 3.2 Electrophoregrams showing the separation of eight reference compounds

Buffer : 100 mM borate (a) pH 9.2 (b) pH 8.0 (c) pH 7.0

3.3 Addition of surfactant

Since the reference compounds were very similar in structure and charge, capillary zone electrophoresis alone could not achieve separation of the eight reference compounds with good resolution. Addition of surfactant may be helpful, thus micellar electrokinetic capillary chromatography was considered.

Surfactant is a surface active agent such as soap or synthetic detergent. It consists of a polar head and a long non-polar tail. At low concentration, surfactant is evenly distributed in buffer solution. At concentration higher than the critical micelle concentration, the surfactants begin to form roughly spherical and dynamic micelles that are at equilibrium with the monomer surfactant molecules in the solution. The hydrophobic tails are orientated toward the center with the polar head groups directed outward. Figure 3.3 showed the basic form of micelle.

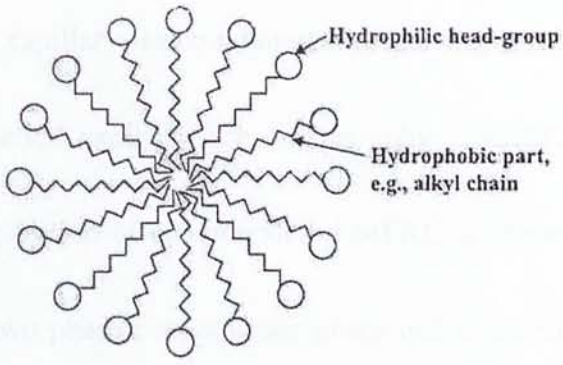


Figure 3.3 Representation of micelle

There are four types of surfactants, namely the anionic, cationic, zwitterionic and nonionic. They are different on the charge of head group. One of the most commonly used anionic surfactant is sodium dodecyl sulfate (SDS). The chemical formula of SDS is $CH_3(CH_2)_{11}OSO_3^-Na^+$ [Figure 3.4]. It has a tail of 12 carbon atoms, attached to a sulfate head group. The critical micelle concentration of SDS is 8.1 [36].

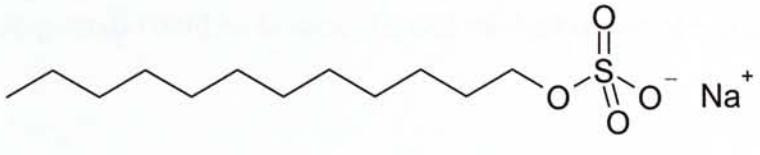


Figure 3.4 Structure of sodium dodecyl sulfate (SDS)

The mode of capillary electrophoresis employing surfactant is known as micellar electrokinetic capillary chromatography (MEKC). The schematic representation of separation of compounds by MEKC is shown in Figure 3.5. The system consists of two phases: an aqueous phase and a micellar phase. SDS is an anionic surfactant, so the micelles migrate towards the anode, which is opposing the electroosmotic flow. Since the electroosmotic flow is stronger than the electrophoretic migration of the micelles under neutral or basic conditions, the SDS micelles migrate toward the cathode at a slow velocity.

The separation of compounds depends on the extent of partitioning into the micelles. Highly polar compounds do not interact with micelles and migrate at the velocity of the electroosmotic flow. Highly hydrophobic compounds have strong interactions with micelles and migrate at the velocity of the micelles. Compounds with intermediate polarities will elute with the velocity in between the two above cases. So, compounds could be separated based on their difference in hydrophobicity.

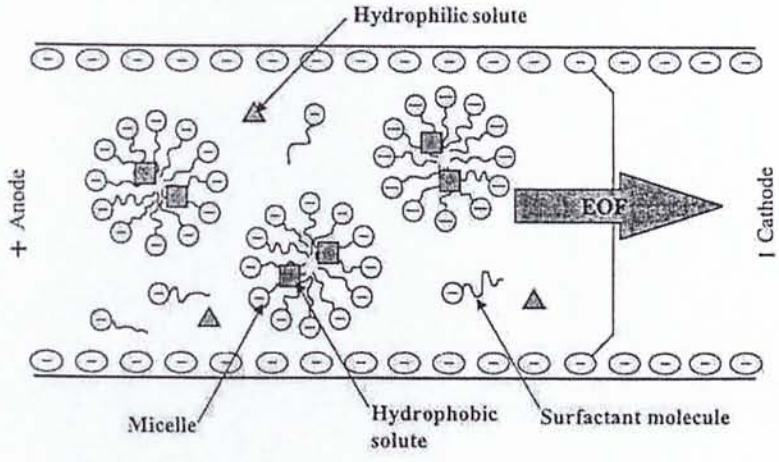


Figure 3.5 Representation of MEKC

Since SDS is able to slow down the migration of compounds, it was chosen as a suitable surfactant for this separation. SDS (50mM) was added to the buffer solution to achieve better separation. The results showed that, all reference compounds could be detected within 27 minutes [Figure 3.6]. (-)-Gallocatecin and (-)-epicatecin could be baseline separated. However, (-)-epigallocatechin and (+)-catechin, as well as (-)-gallocatechin gallate and (-)-epigallocatechin gallate, could not be separated. So, SDS was considered to be essential for the separation, but its concentration should be adjusted to achieve a better separation.

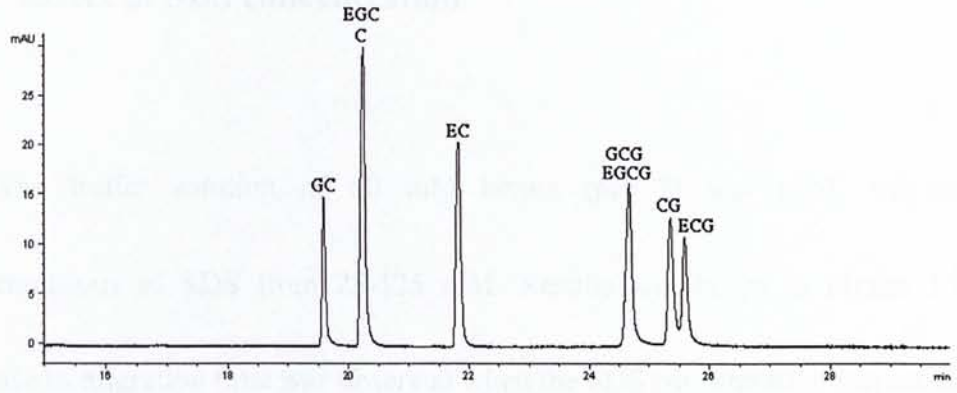


Figure 3.6 Electrophoregram showing the separation of eight reference compounds

Buffer : 50mM borate solution, 50 mM SDS (pH 7)

3.4 Effect of SDS concentration

The buffer solution of 50 mM borate (pH 7) was used, varying the concentrations of SDS from 25-125 mM. Results are shown in Figure 3.7. An increase in migration time was observed when the SDS concentration increased. This is because when the SDS concentration is increased, the formation of micelles in the buffer is enhanced and the analytes would have a greater chance to be incorporated into the micelles, thus prolonging the migration time. The separation was also improved as the SDS concentration was increased. (+)-Catechin, (-)-epicatechin, (-)-epigallocatechin and (-)-catechin gallate are smaller in size and thus more hydrophilic compared to other reference compounds having gallate groups, so the migration times of these four reference compounds were shorter. When the SDS concentration reached 100 mM, separation of the eight reference compounds could be achieved [Figure 3.8]. However, the separation of (-)-gallocatechin gallate and (-)-epigallocatechin gallate was not satisfactory, it was necessary to further improved in order to obtain baseline separation of all eight reference compounds.

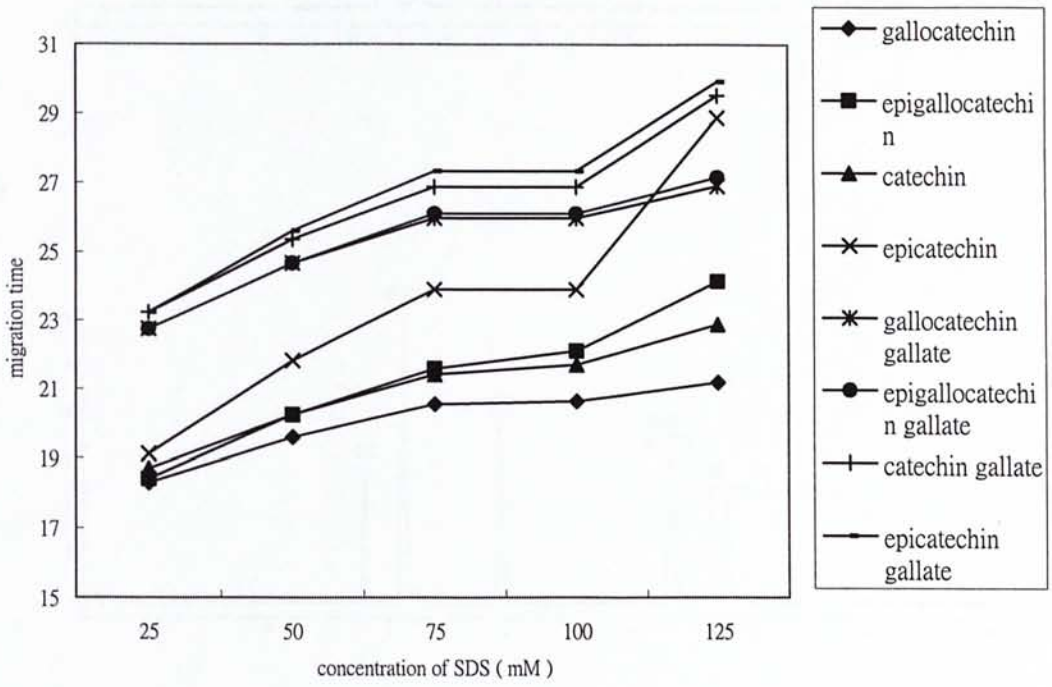
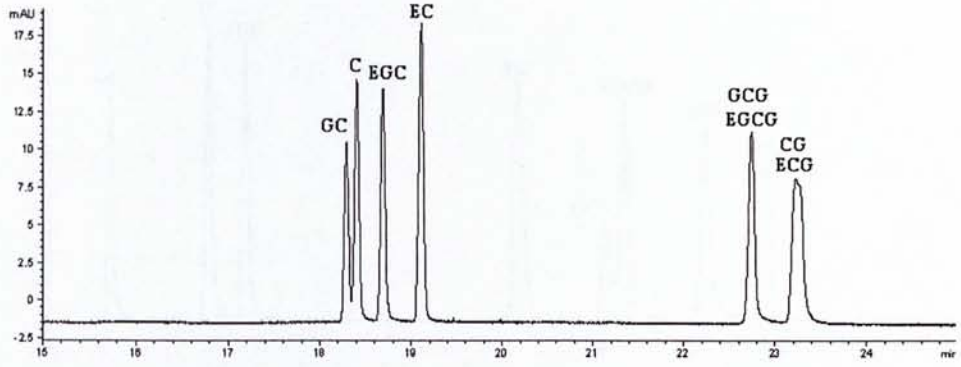


Figure 3.7 Effect of SDS on migration time

Buffer : 50mM borate and SDS (pH 7)

(a)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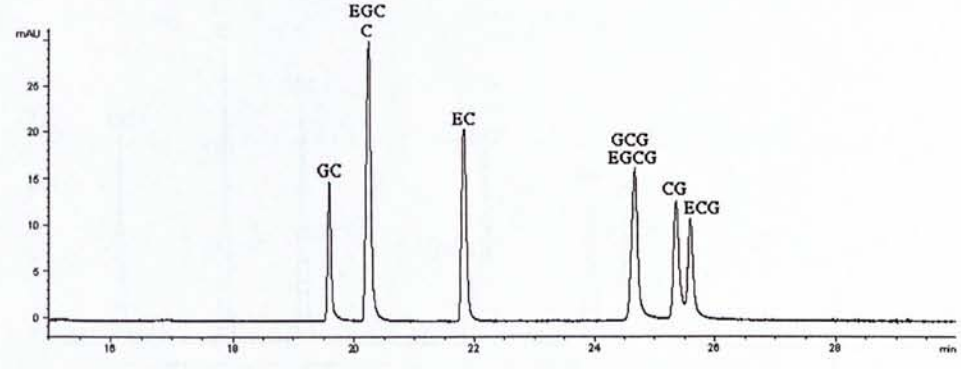
(b)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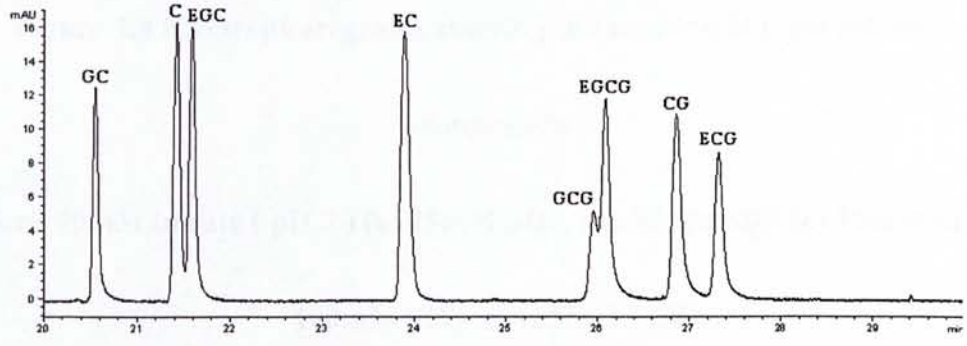
(c)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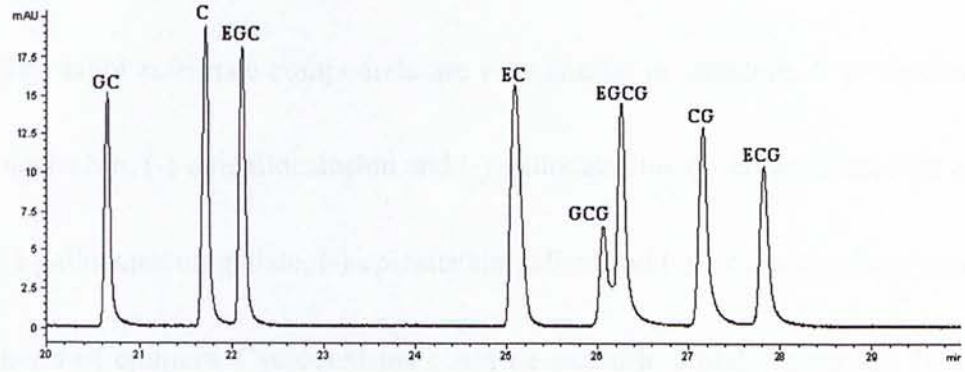
(d)



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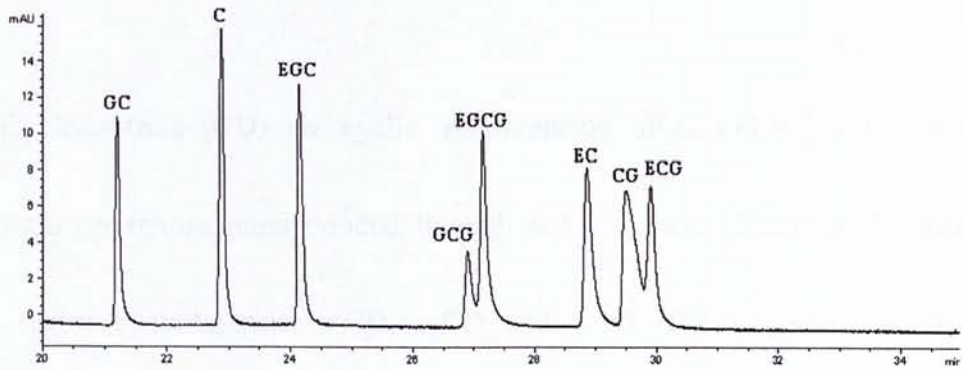


Figure 3.8 Electrophoregrams showing the sepation of eight reference compounds

Buffer : 50mM borate (pH 7) (a) 25mM SDS, (b) 50mM SDS (c) 75mM SDS (d) 100mM SDS (e) 125mM SDS

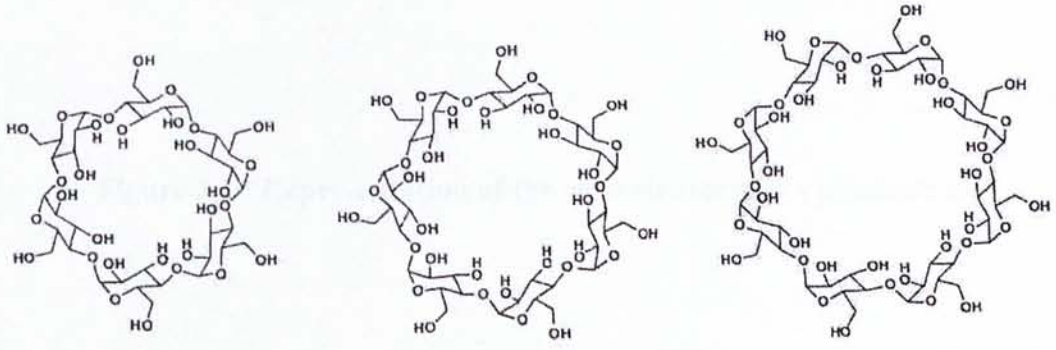
3.5 Addition of cyclodextrins

The eight reference compounds are very similar in structure. (+)-Catechin and (-)-epicatechin, (-)-epigallocatechin and (-)-gallocatechin, (-)-epigallocatechin gallate and (-)-gallocatechin gallate, (-)-epicatechin gallate and (-)-catechin gallate belong to four pairs of epimers. Cyclodextrins could be useful in chiral separation. Therefore cyclodextrins were added to the buffer system in order to test if better separation can be obtained.

Cyclodextrins (CD) are cyclic, non-reducing oligosaccharides consisting of D-(+)-glucopyranose units bonded through α-1,4 linkage [Figure 3.9]. There are three major cyclodextrins: α-CD, β-CD and γ-CD. The physical and chemical properties of the three CDs were shown in Table 3.3.

	α-CD	β-CD	γ-CD
Glucose units	6	7	8
Internal diameter	0.47-0.53	0.60-0.65	0.75-0.83
Depth of cavity	0.79	0.79	0.79
pKa value	12.3	12.2	12.1
Water solubility	145	18.5	232
MW	972	1135	1297
Approximate volume of cavity	174	262	427

Table 3.3 Properties of the α-CD, β-CD and γ-CD



α-CD β-CD γ-CD

Figure 3.9 Structure of α-CD, β-CD and γ-CD

In CDs, the sugar is of $^4C_1$ conformation. The entire molecule is of a toroidal truncated cone structure [Figure 3.10]. The surface of the internal cavity is hydrophobic in nature, and hydroxyl groups are on the external surface. Because of this characteristic of CD, it can form inclusion complex with a wide range of organic and inorganic molecules [37].

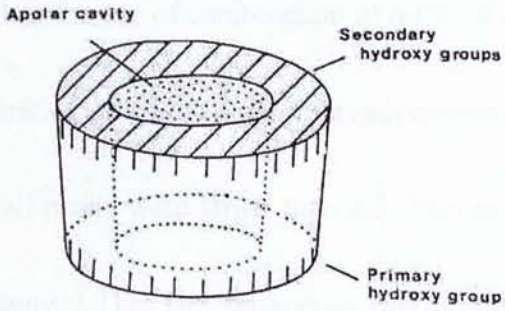


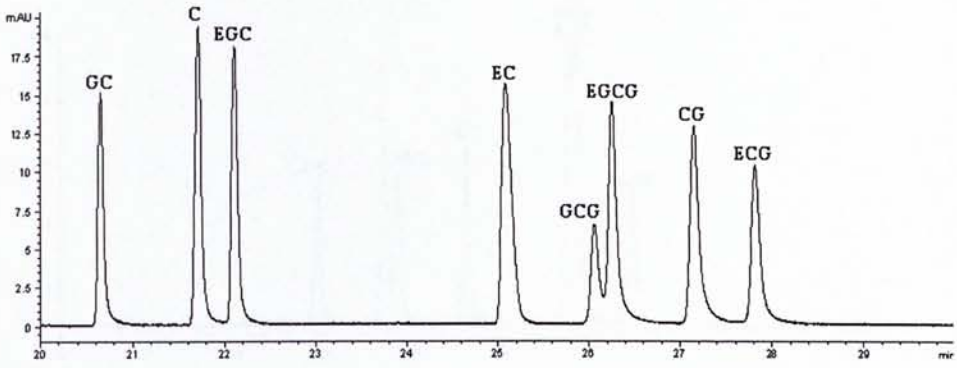
Figure 3.10 Representation of the cone structure of cyclodextrin

The inclusion complex is formed when the CDs includes a molecule having a size compatible with the dimension of the cavity. The molecule can be totally or partially included in the CD's cavity. The inclusion processes is medicated by physical force, no covalent bonding is involved. The size of the CD's cavity is suitable for molecules with two benzene ring or even larger compounds. So CDs are suitable host for flavan-3-ols. The strength of inclusion complex depends on a lot of

factors, such as steric compatibility, hydrophobic interaction, van der Waals interaction and hydrogen bonding.

α-, β-, γ-CD as well as their mixtures were added to the buffer system of 50 mM borate and 100 mM SDS. The results showed that, when α-CD, β-CD and γ-CD were used alone, the separation was not satisfactory. But the use of the combination of β-CD and γ-CD as well as the use of combination of α-CD, β-CD and γ-CD provided better results. The separation of all the eight reference compounds could be achieved and the resolutions of all peaks were larger than 1.5. That means all peaks could be baseline separated [Figure 3.11]. The separation was improved because the eight reference compounds have different size and orientation, these differences made them fit into the cyclodextrins by different extent. The eight reference compounds thus reach the detector with different migration time.

(a)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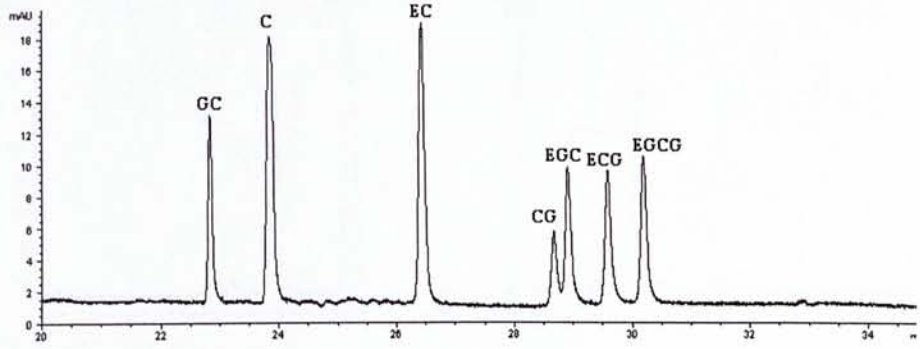
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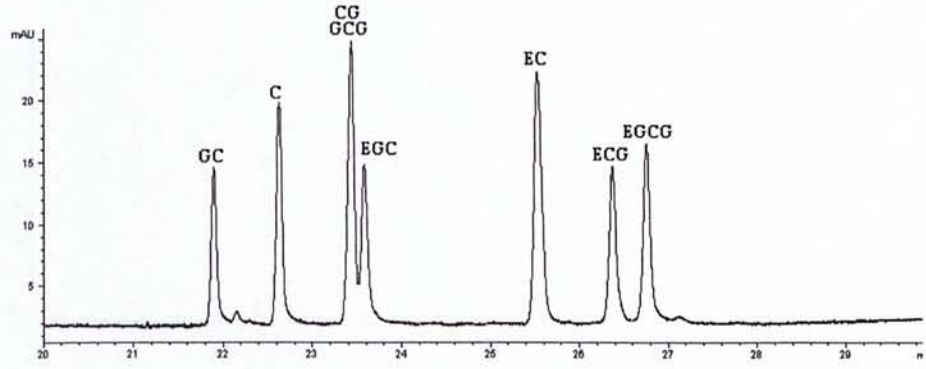
(c)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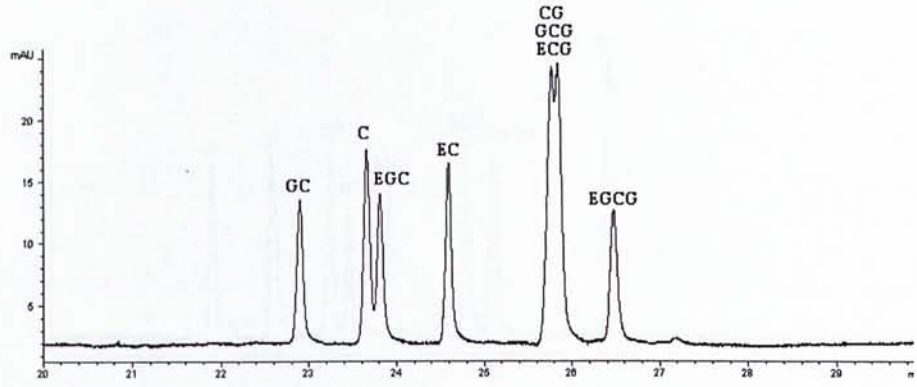
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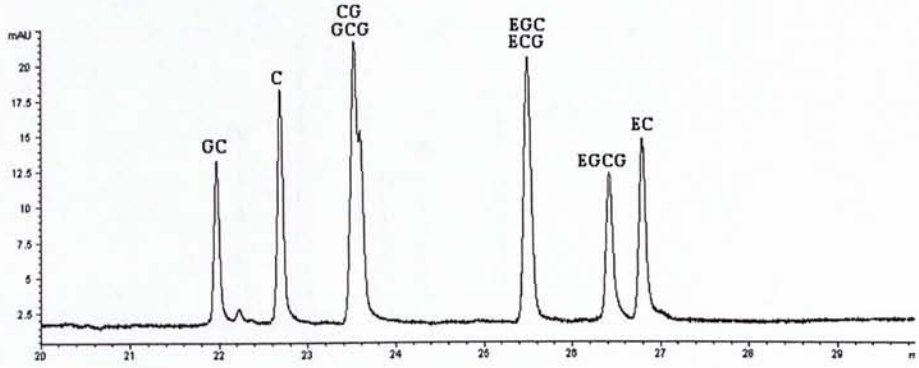
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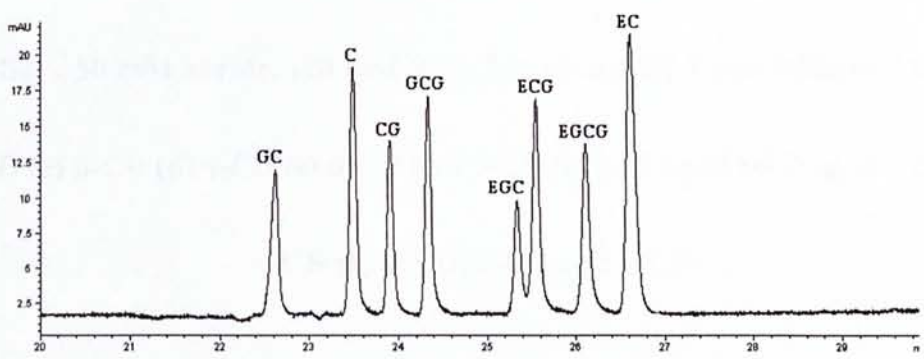
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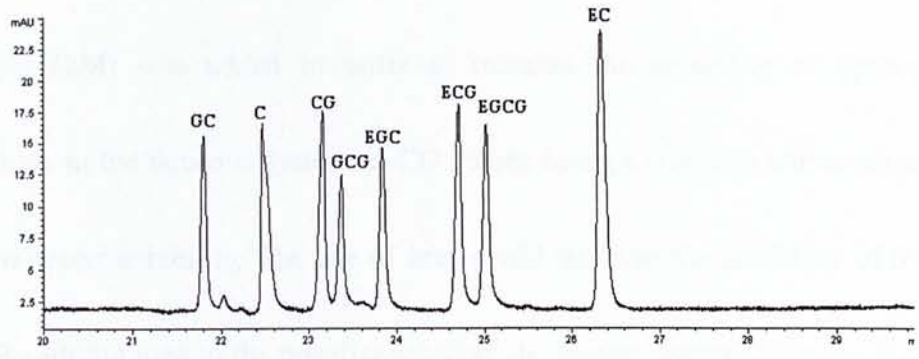
(g)



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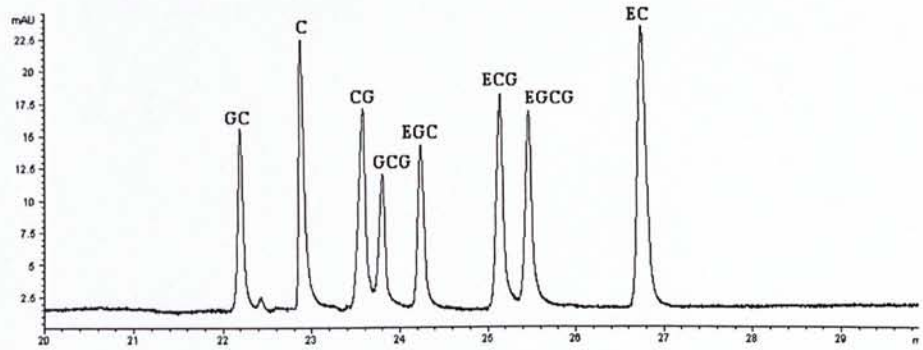


Figure 3.11 Electrophoregrams showing the separation of the eight reference compounds

Buffer : 50 mM borate, 100 mM SDS, 2 M urea (pH 7) (a) without CD (b) α-CD (c) β-CD (d) γ-CD (e) α-CD and β-CD (f) α-CD and γ-CD (g) β-CD and γ-CD (h) α-CD, β-CD and γ-CD

3.6 Urea

Urea (2M) was added in order to increase the solubility of hydrophobic compounds in the aqueous system. β-CD posses being a common chiral reagent has very low water solubility. The use of urea could improve the solubility of β-CD in water. By adding urea to the micellar solution, the electro-osmotic velocity is slightly reduced, whereas the migration velocity of the micelle is slower. Urea is also effective in improving peak shape [38].

3.7 Addition of Organic modifier

An organic solvent miscible with water can be used as an additive to the micellar solution to manipulate the capacity factor or selectivity. Yet a high concentration of organic modifier should be avoided because it will lead to the breakdown of micellar structures. In general, the maximum content of organic modifier should not exceed 20%. In capillary electrophoresis, methanol and acetonitrile are most commonly used as the organic modifier. They can reduce the electro-osmotic velocity and expand the migration time window [39].

Ten percent Methanol and acetonitrile were tried in the buffer solution, respectively [Figure 3.12 and 3.13]. The results showed that the addition of these organic solvents could not yield a better separation. Organic modifier was therefore considered to be not suitable for flavan-3-ols separation.

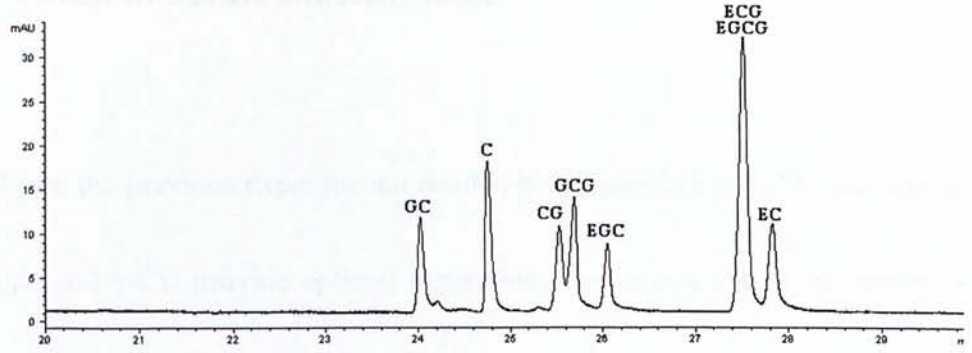


Figure 3.12 Electrophoregram showing the separation of the eight reference compounds

Buffer : 50mM borate, 100 mM SDS, 5 mM β-CD, 5 mM γ-CD, 2M urea 10% methanol (pH 7)

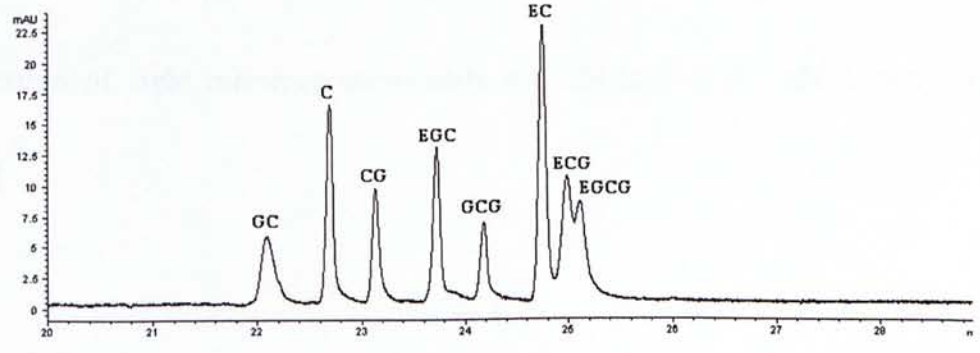


Figure 3.13 Electrophoregram showing the separation of the eight reference compounds

Buffer : 50mM borate, 100 mM SDS, 5 mM β-CD, 5 mM γ-CD, 2 M urea 10% acetonitrile (pH 7)

3.8 Effect of borate concentration

From the previous experimental results, it was concluded that borate buffer with SDS, β- and γ-CD provide optimal separation. The concentration of borate, β- and γ-CD was further optimized.

In order to study the effect of borate concentration, buffers containing borate concentrations of 10-50 mM were investigated. The results are shown in Figure 3.14. It was observed that when the borate concentration was increased, the migration time of all reference compounds became longer. At concentrations below 30 mM, the separation of all eight reference compounds could not be achieved. A baseline separation of eight reference compounds was obtained at 40 mM borate [Figure 3.15].

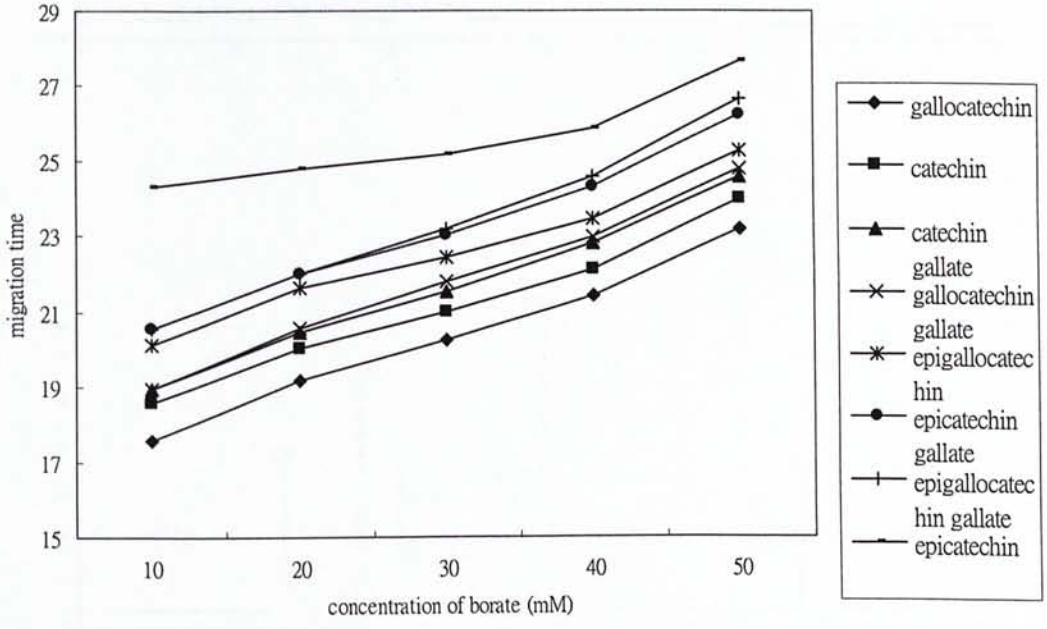
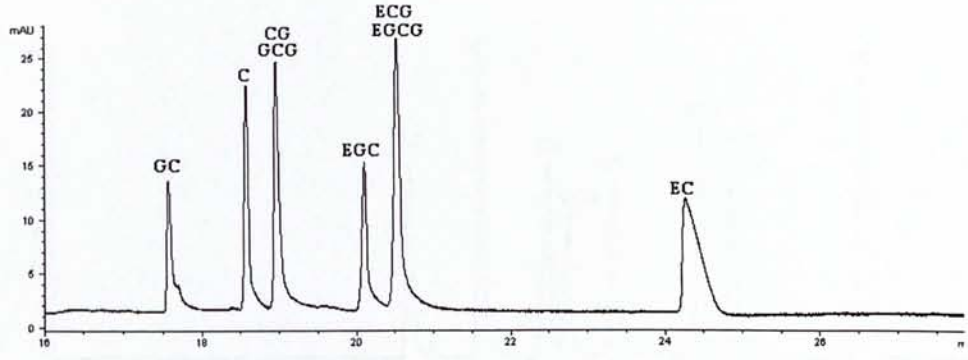


Figure 3.14 Effect of borate on migration time

Buffer : 100 mM SDS, 5 mM β-CD, 5 mM γ-CD, 2M urea, borate (pH 7)

(a)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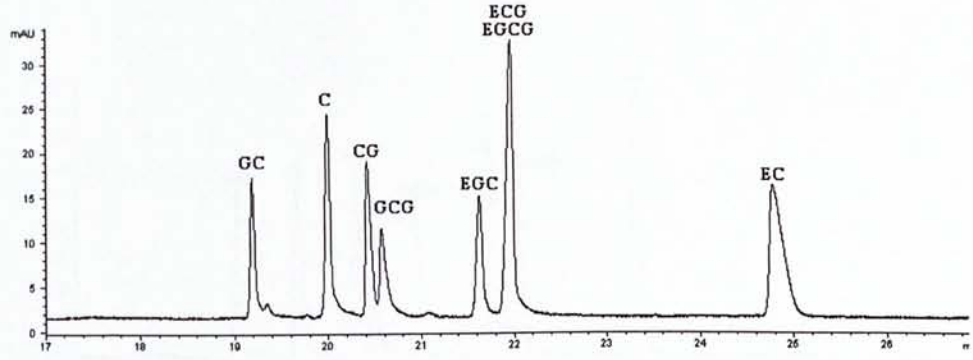
(b)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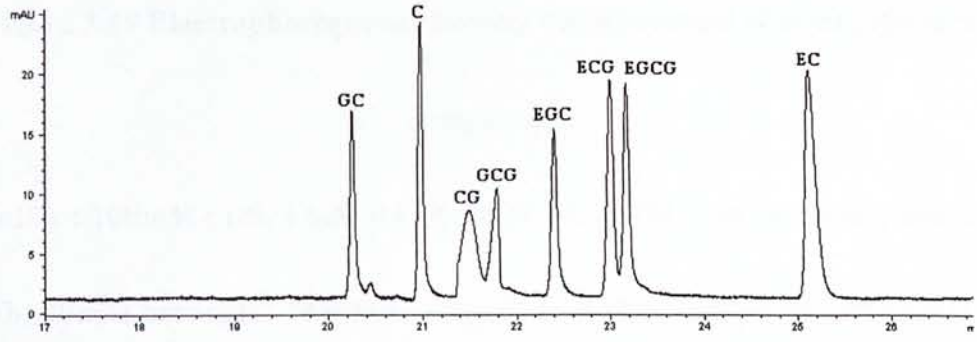
(c)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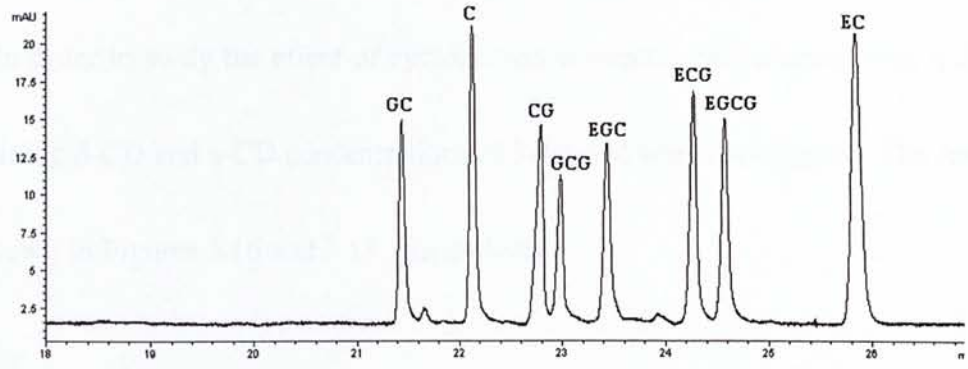
EC

(d)



(e)

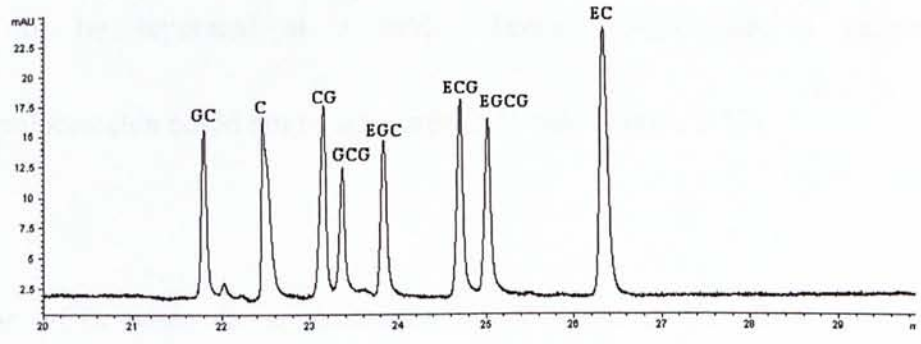


Figure 3.15 Electrophoregrams showing the separation of eight reference compounds

Buffer : 100mM SDS, 5 mM β-CD, 5mM γ-CD, 2M urea (a) 10 mM borate (b) 20mM borate (c) 30 mM borate (d) 40 mM borate (e) 50 mM borate

3.9 Effect of cyclodextrin concentration

In order to study the effect of cyclodextrin concentration on separation, buffers containing β-CD and γ-CD concentrations of 3-10 mM were investigated. The results are shown in Figures 3.16 and 3.18, respectively.

For β-CD, when the concentration range increased from 3-10 mM, optimal separation was observed at 5 mM. (-)-Catechin gallate and (-)-gallocatechin gallate could not be separated at 3 mM, whereas (-)-gallocatechin gallate and (-)-epigallocatechin could not be separated at 10 mM [Figure 3.17].

For γ-CD, when the concentration range increased from 3-10 mM, optimal separation was also observed at 5 mM. (-)-Catechin gallate and (-)-gallocatechin gallate, (-)-epicatechin gallate and (-)-epigallocatechin gallate could not be baseline separated at 3 mM, whereas (-)-gallocatechin gallate and (-)-epigallocatechin could not be baseline separated at 10 mM [Figure 3.19].

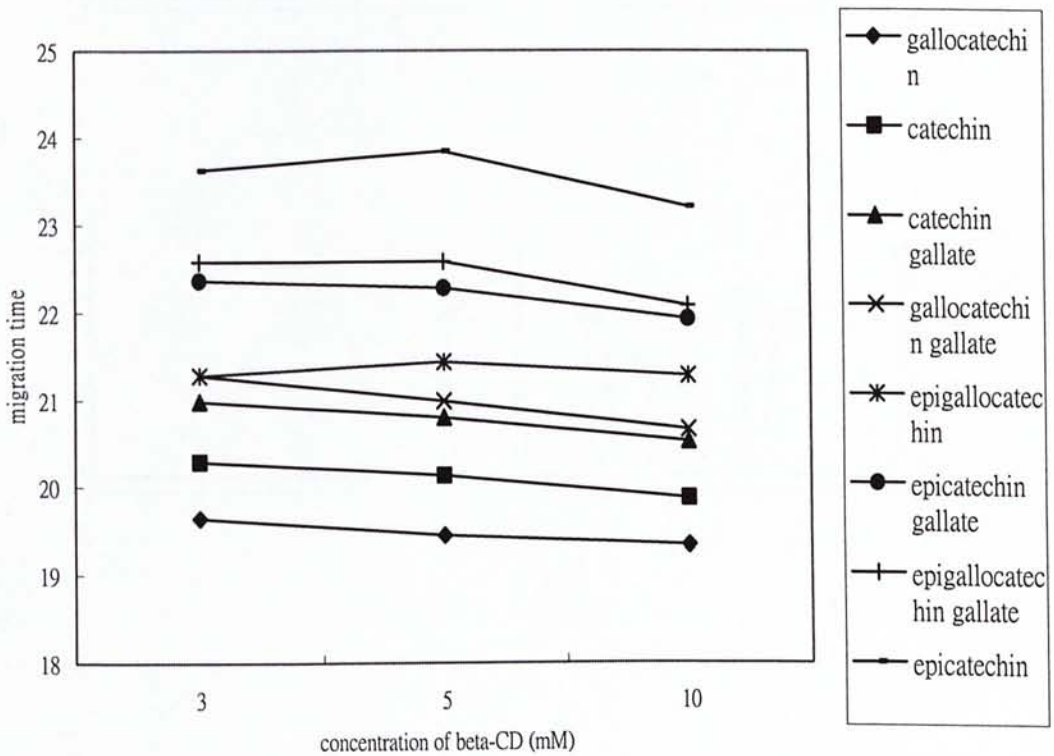
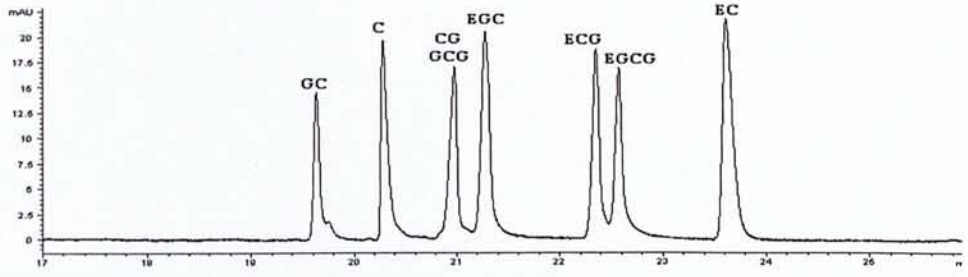


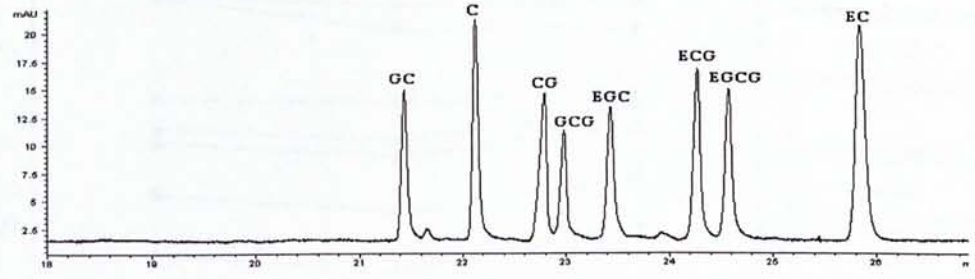
Figure 3.16 Effect of β-CD on migration time

Buffer : 40mM borate, 100 mM SDS, 5 mM γ-CD, 2M urea, β-CD (pH 7)

(a)



(b)



(c)

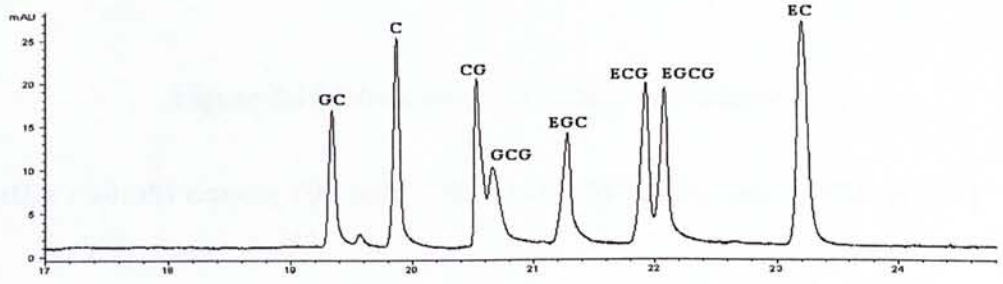


Figure 3.17 Electrophoregrams showing the separation of eight reference compounds

Buffer : 40 mM borate, 100 mM SDS, 5 mM γ-CD, 2M urea (pH 7)

(a) 3 mM β-CD (b) 5 mM β-CD (c) 10 mM β-CD

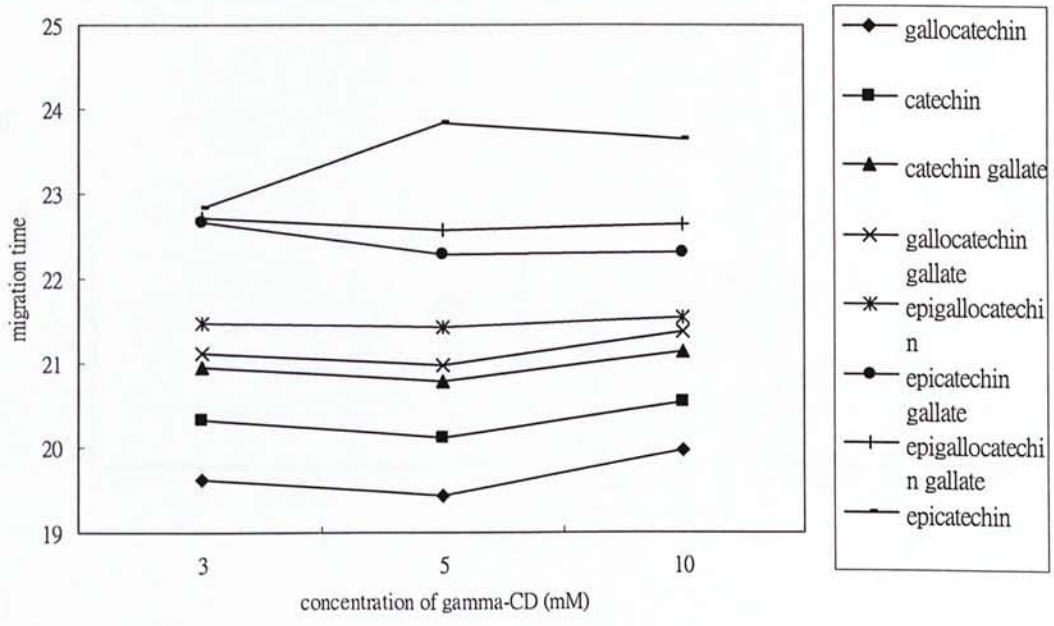
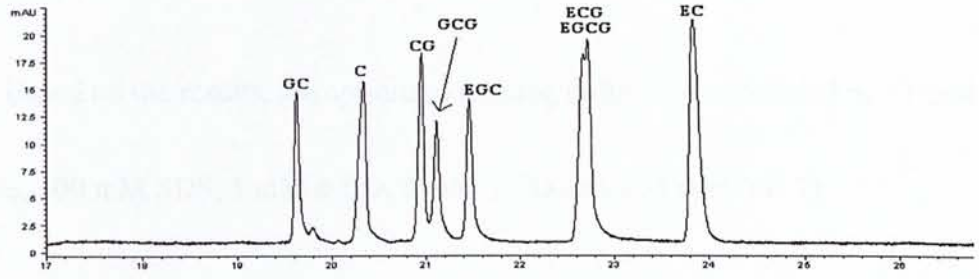


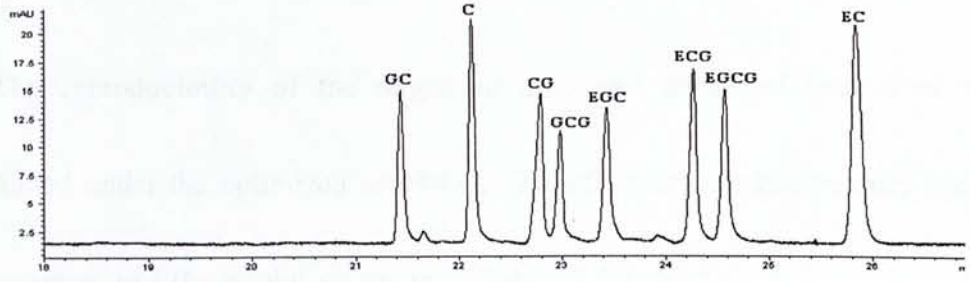
Figure 3.18 Effect of γ-CD on migration time

Buffer : 40mM borate, 100 mM SDS, 5 mM β-CD, 2M urea, γ-CD (pH 7)

(a)



(b)



(c)

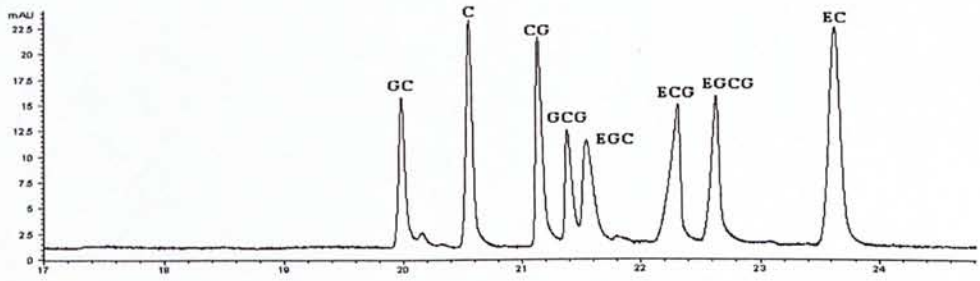


Figure 3.19 Electrophoregrams showing the separation of eight reference compounds

Buffer : 40 mM borate, 100 mM SDS, 5 mM β-CD, 2M urea (pH 7)

(a) 3 mM γ-CD (b) 5 mM γ-CD (c) 10 mM γ-CD

3.10 Optimized condition

Based on the results, the optimized running buffer was concluded as 40 mM borate, 100 mM SDS, 5 mM β-CD, 5 mM γ-CD and 2 M urea (pH 7).

3.11 Reproducibility of the method

The reproducibility of the migration time and integrated peak area were determined under the optimized condition. The reference compounds were injected for five times, and the results are shown in Tables 3.4 and 3.5.

Reference compounds	Average migration time	Relative standard deviation
(-)-gallocatechin	19.3534	0.346 %
(+)-catechin	19.9436	0.338 %
(-)-catechin gallate	20.5996	0.343 %
(-)-gallocatechin gallate	20.7896	0.389 %
(-)-epigallocatechin	20.9884	0.325 %
(-)-epicatechin gallate	21.9264	0.423 %
(-)-epigallocatechin gallate	22.0594	0.517 %
(-)-epicatechin	23.0154	0.337 %

Table 3.4 Relative standard deviation of migration time of the reference compounds

Reference compounds	Average integrated peak area	Relative standard deviation
(-)-gallocatechin	52.1496	1.673%
(+)-catechin	80.6237	0.962 %
(-)-catechin gallate	64.9052	4.771 %
(-)-gallocatechin gallte	41.5253	2.693 %
(-)-epigallocatechin	62.1879	2.351 %
(-)-epicatechin gallate	56.4277	0.996 %
(-)-epigallocatechin gallate	67.4505	4.168 %
(-)-epicatechin	108.7123	3.490 %

Table 3.5 Relative standard deviation of integrated peak area of the reference compounds

From the results shown in the tables above, the relative standard deviations of 5 replicates were satisfactory both for the migration time and integrated peak area. So, the proposed method is concluded to be satisfactory. The method was then applied to the analysis of samples of grape seed extract.

3.12 Quantitative analysis of reference compounds

Calibration curves of the eight reference compounds were obtained. Calibration of (+)-catechin was shown below [Figure 3.20]. Other calibration curves show similar results. All calibration curves showed satisfactory linearity. Regression equation, correlation coefficient and detection limits of the eight reference compounds were shown in Table 3.6 and 3.7.

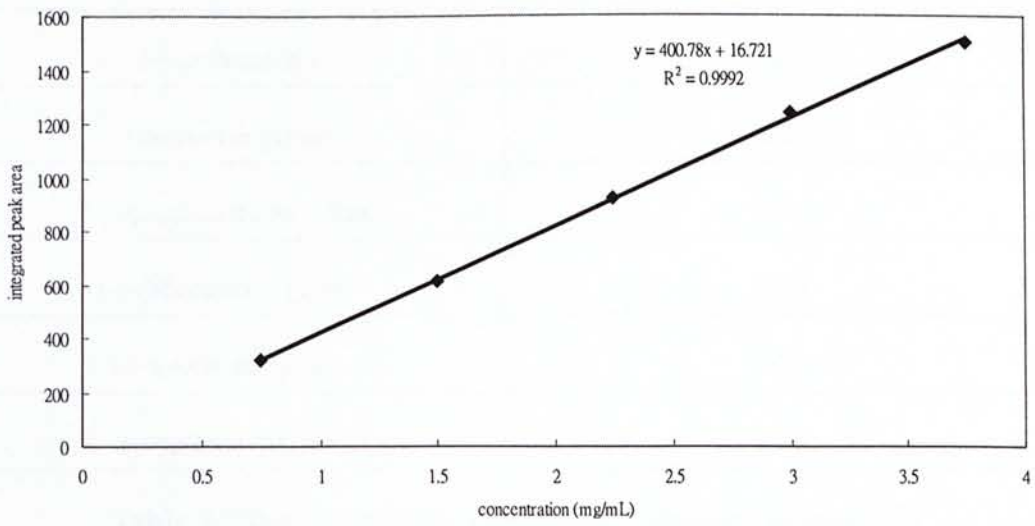


Figure 3.20 Calibration curve of (+)-catechin

Reference compounds	Regression equation	Correlation coefficient
(+)-catechin	y=400.78x+ 16.721	0.9992
(-)-epicatechin	y=349.64x+ 62.657	0.9982
(-)-gallocatechin	y=409.5x+ 55.343	0.9991
(-)-catechin gallate	y=380.2x+ 58.527	0.9992
(-)-epigallocatechin gallate	y=303.87x+37.414	0.9985
(-)-gallocatechin gallate	y=103.44x+ 0.1871	0.9998
(-)-epicatechin gallate	y=330.45x+ 7.927	0.9992
(-)-epigallocatechin	y=428.81x+ 0.3298	0.9988

Table 3.6 Regression equation and correlation coefficient of the eight reference compounds

Reference compounds	Detection limits (mg/ml)
(+)-catechin	0.010
(-)-epicatechin	0.039
(-)-gallocatechin	0.035
(-)-catechin gallate	0.037
(-)-epigallocatechin gallate	0.024
(-)-gallocatechin gallate	0.001
(-)-epicatechin gallate	0.004
(-)-epigallocatechin	0.002

Table 3.7 Detection limits of the eight reference compounds

3.13 Application of the CE method in grape seed products

The developed method was then applied to the samples of grape seed extract. The electrophoregram obtained from each samples are shown below [Figure 3.21-3.27].

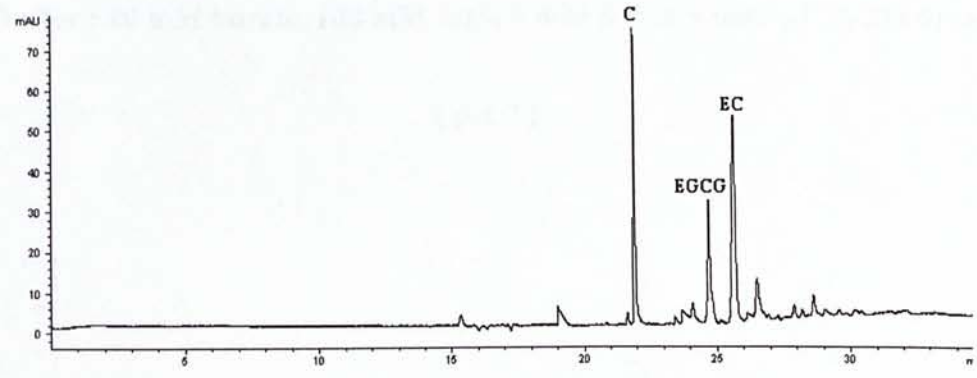


Figure 3.21 Electrophoregram of sample from Future Ceuticals

Buffer : 40 mM borate, 100 mM SDS, 5 mM β-CD, 5 mM γ-CD, 2M urea (pH 7)

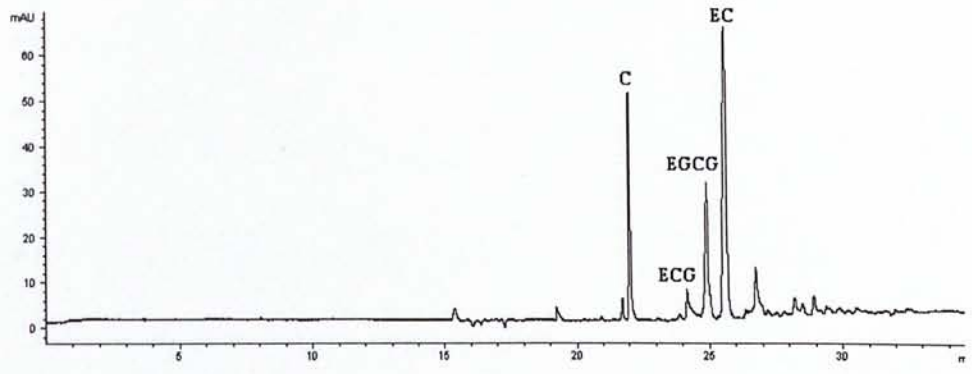


Figure 3.22 Electrophoregram of sample from DNP International Co. Inc

Buffer : 40 mM borate, 100 mM SDS, 5 mM β-CD, 5 mM γ-CD, 2M urea

(pH 7)

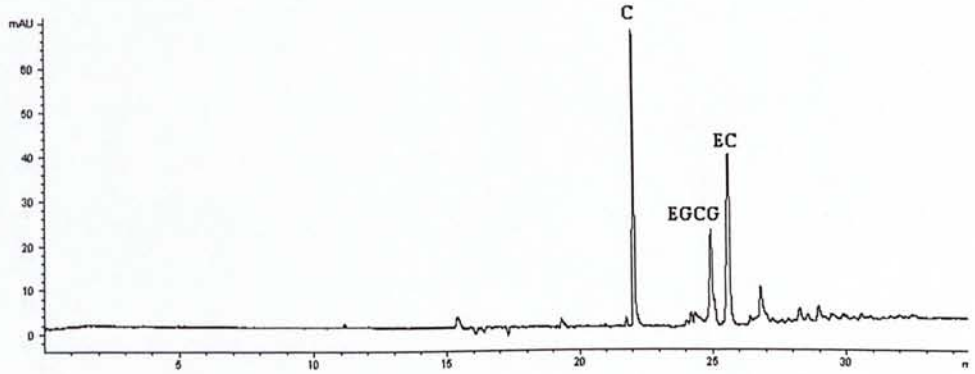


Figure 3.23 Electrophoregram of sample from AMAX Nutritional Inc

Buffer : 40 mM borate, 100 mM SDS, 5 mM β-CD, 5 mM γ-CD, 2M urea

(pH 7)

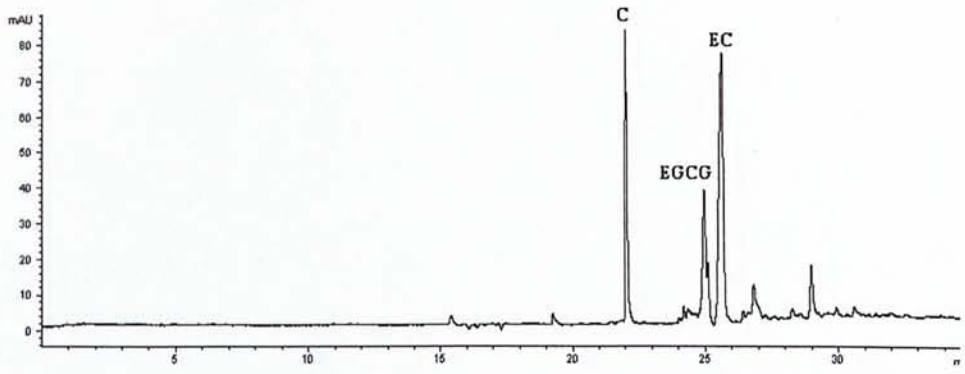


Figure 3.24 Electrophoregram of sample from Scanadinavian Formulars Chemical and Ingredients Division

Buffer : 40 mM borate, 100 mM SDS, 5 mM β-CD, 5 mM γ-CD, 2M urea (pH 7)

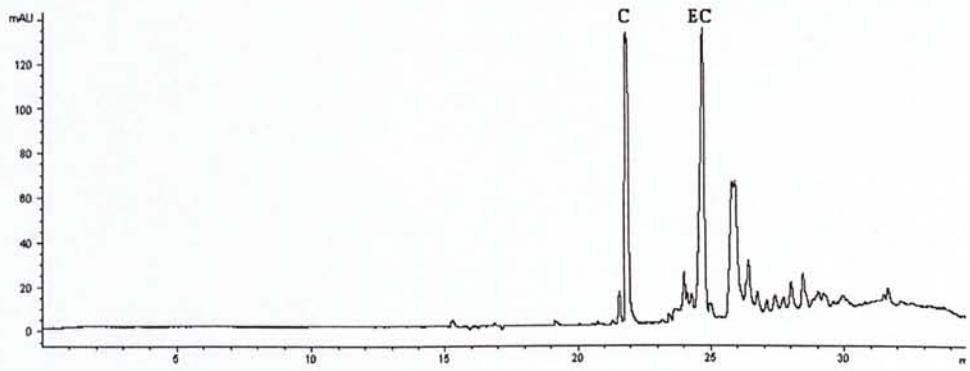


Figure 3.25 Electrophoregram of sample from Polyphenolics

Buffer : 40 mM borate, 100 mM SDS, 5 mM β-CD, 5 mM γ-CD, 2M urea (pH 7)

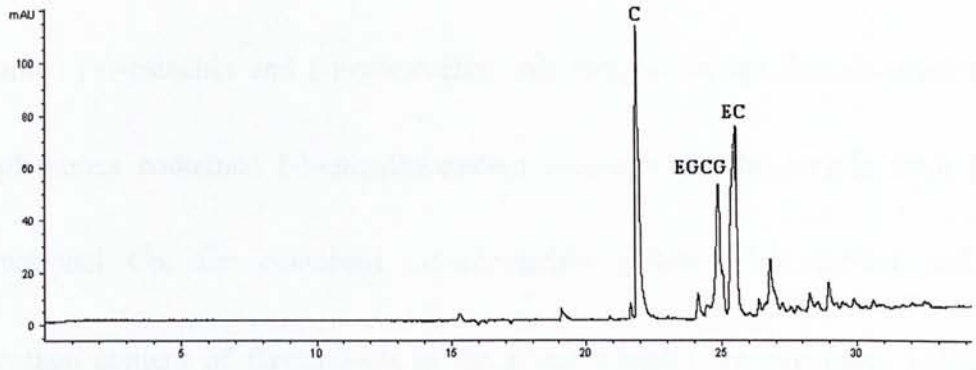


Figure 3.26 Electrophoregram of sample from Trusperity USA Inc

Buffer : 40 mM borate, 100 mM SDS, 5 mM β-CD, 5 mM γ-CD, 2M urea

(pH 7)

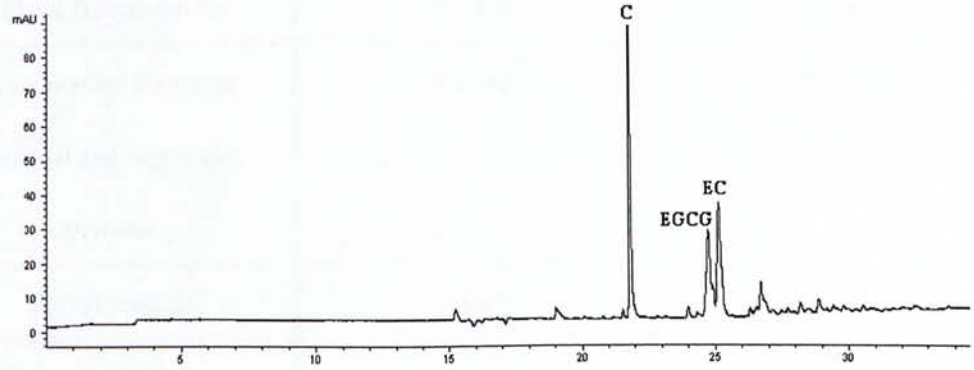


Figure 3.27 Electrophoregram of sample from Neutratech

Buffer : 40 mM borate, 100 mM SDS, 5 mM β-CD, 5 mM γ-CD, 2M urea

(pH 7)

From the electrophoregramS obtained, it was shown that all seven samples contained (+)-catechin and (-)-epicatechin. All samples except that obtained from Polyphenolics contained (-)-epigallocatechin gallate. Only the sample from DNP International Co. Inc contained (-)-epicatechin gallate. The amount and the percentage content of flavan-3-ols in the seven samples are calculated using the respective calibration curves and shown in the Tables 3.8-3.11.

Samples	**Amount of (+)-catechin**	**Conent (%)**
Future Ceuticals	0.8328	0.0833
DNP International Co. Inc	0.5259	0.0526
AMAX Nutritional Inc	0.7446	0.0745
Scandinavian Formulas Chemical and Ingredients Division	0.9792	0.0979
Polyphenolics	3.1280	0.3128
Trusperity USA inc	2.6372	0.2637
Nutratech	1.1219	0.1122

Table 3.8 Quantitative determination of the (+)-catechin in the seven samples

Samples	Amount of (-)-epicatechin	Conent (%)
Future Ceuticals	1.5480	0.1548
DNP International Co. Inc	1.8804	0.1880
AMAX Nutritional Inc	1.0991	0.1099
Scandinavian Formulas Chemical and Ingredients Division	2.7956	0.2796
Polyphenolics	4.9033	0.4903
Trusperity USA inc	1.8211	0.1821
Nutratech	1.2149	0.1215

Table 3.9 Quantitative determination of the (-)-epicatechin in the seven samples

Samples	Amount of (-)-epigallocatechin gallate	Conent (%)
Future Ceuticals	0.6720	0.0672
DNP International Co. Inc	0.6971	0.0697
AMAX Nutritional Inc	0.5553	0.0555
Scandinavian Formulas Chemical and Ingredients Division	1.0345	0.1035
Polyphenolics	----------	----------
Trusperity USA inc	2.1106	0.2111
Nutratech	0.7381	0.0738

Table 3.10 Quantitative determination of the (-)-epigallocatechin gallate in the seven samples

Samples	Amount of (-)-epicatechin gallate	Conent (%)
DNP International Co. Inc	0.1864	0.0186

Table 3.11 Quantitative determination of the (-)-epicatechin gallate in the seven samples

Chapter 4

Conclusion

In this study, an analytical method was developed for the determination of flavan-3-ols consistuents in grape seed extract by capillary electrohporesis. Under the optimal condition, eight reference compounds could be resolved in the electrophoregram. Eight reference compounds, namely, (+)-catechin, (-)-epicatechin, (-)-catechin gallate, (-)-epicatechin gallate, (-)-gallocatechin, (-)-epigallocatechin, (-)-gallocatechin gallate, (-)-epigallocatechin gallate were used in the present study.

The use of borate buffer alone gave unsatisfactory results. The eight reference compounds could not be separated and the resolution was poor. By the addition of surfactant (SDS), the separation was greatly improved. The eight reference compounds could be separated and the resolution had significantly improved. In order to obtain baseline separation of all reference compounds, cyclodextrin was added. By optimizing the concentrations of surfactant and cyclodextrin, the eight reference compounds could be baseline separated. The reproducibility of migration time and integrated peak area was then estimated. The results were satisfactory, indicating that the method is suitable for qualitative and quantitative analysis of

flavan-3-ols.

The method was then applied to the analysis of grape seed extract from seven commercial sources. The analysis could be complete within 30 minutes. (+)-Catechin and (-)-epicatechin were found in all seven samples, (-)-epigallocatechin gallate and (-)-epicatechin gallate could be detected only in some of the samples. The remaining reference compounds were not detected.

It is concluded that the proposed method is feasible in the separation of the major flavan-3-ols in grape seed extract. It has the advantages of short analysis time, high resolution, small volume of sample and buffer consumption. This made it a good alternative and complementary method from HPLC analysis.

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